(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
7 April 2005 (07.04.2005)

PCT

(10) International Publication Number
WO 2005/030251 A1

(51) International Patent Classification[7]: A61K 39/395, C12N 5/06, A61P 37/04

(21) International Application Number:
PCT/US2004/030895

(22) International Filing Date:
22 September 2004 (22.09.2004)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
60/505,248 22 September 2003 (22.09.2003) US
60/577,195 4 June 2004 (04.06.2004) US

(71) Applicant *(for all designated States except US)*: **XCYTE THERAPIES, INC.** [US/US]; 1124 Columbia Street, Suite 130, Seattle, Washington 98104 (US).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: **BERENSON, Ronald J.** [US/US]; P.O. Box 1597, Mercer Island, Washington 98040 (US). **FROHLICH, Mark, Walter** [US/US]; 7326 78th Avenue Southeast, Mercer Island, Washington 98040 (US).

(74) Agents: **URVATER, Julie, A.** et al.; Seed Intellectual Property Law Group PLLC, Suite 6300, 701 Fifth Avenue, Seattle, WA 98104-7092 (US).

(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NA, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RU, SC, SD, SE, SG, SK, SL, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, YU, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IT, LU, MC, NL, PL, PT, RO, SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:
— *with international search report*
— *before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) Title: COMPOSITIONS AND METHODS TO ACCELERATE HEMATOLOGIC RECOVERY

(57) Abstract: The present invention relates generally to methods for activating and expanding T cells, and more particularly, to methods for restoring hematologic function and/or accelerating hematologic recovery in patients by administering said T cells. Compositions of cells activated and expanded by the methods herein are further provided.

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COMPOSITIONS AND METHODS TO ACCELERATE HEMATOLOGIC RECOVERY

BACKGROUND OF THE INVENTION

Field of the Invention

The present invention relates generally to methods for restoring hematologic function and/or accelerating hematologic recovery in a subject. The present provides methods for stimulating and activating T cells and to methods to activate and expand T cells to high numbers. The present invention also relates to compositions of expanded T cells and to methods of using said T cells. In particular, the present invention relates to methods of accelerating hematologic recovery by administering to patients T cells expanded according to the methods described herein.

Description of the Related Art

The T cell antigen receptor (TCR) is a multisubunit immune recognition receptor that associates with the CD3 complex and binds to peptides presented by the major histocompatibility complex (MHC) class I and II proteins on the surface of antigen-presenting cells (APCs). Binding of TCR to the antigenic peptide on the APC is the central event in T cell activation, which occurs at an immunological synapse at the point of contact between the T cell and the APC.

To sustain T cell activation, T lymphocytes typically require a second co-stimulatory signal. Co-stimulation is typically necessary for a T helper cell to produce sufficient cytokine levels that induce clonal expansion. Bretscher, *Immunol. Today 13*:74, 1992; June *et al., Immunol. Today 15*:321, 1994. The major co-stimulatory signal occurs when a member of the B7 family ligands (CD80 (B7.1) or CD86 (B7.2)) on an activated antigen-presenting cell (APC) binds to CD28 on a T cell.

Methods of stimulating the expansion of certain subsets of T cells have the potential to generate a variety of T cell compositions useful in immunotherapy.

Successful immunotherapy can be aided by increasing the reactivity and quantity of T cells by efficient stimulation.

The various techniques available for expanding human T cells have relied primarily on the use of accessory cells and/or exogenous growth factors, such as interleukin-2 (IL-2). IL-2 has been used together with an anti-CD3 antibody to stimulate T cell proliferation, predominantly expanding the $CD8^+$ subpopulation of T cells. Both APC signals are thought to be required for optimal T cell activation, expansion, and long-term survival of the T cells upon re-infusion. The requirement for MHC-matched APCs as accessory cells presents a significant problem for long-term culture systems because APCs are relatively short-lived. Therefore, in a long-term culture system, APCs must be continually obtained from a source and replenished. The necessity for a renewable supply of accessory cells is problematic for treatment of immunodeficiencies in which accessory cells are affected. In addition, when treating viral infection, if accessory cells carry the virus, the cells may contaminate the entire T cell population during long-term culture.

In the absence of exogenous growth factors or accessory cells, a co-stimulatory signal may be delivered to a T cell population, for example, by exposing the cells to a CD3 ligand and a CD28 ligand attached to a solid phase surface, such as a bead. *See* C. June, *et al.* (U.S. Patent No. 5,858,358); C. June *et al.* WO 99/953823. While these methods are capable of achieving therapeutically useful T cell populations, increased robustness and ease of T cell preparation remain less than ideal.

In addition, the methods currently available in the art have not focused on short-term expansion of T cells or obtaining a more robust population of T cells and the beneficial results thereof. Furthermore, the applicability of expanded T cells has been limited to only a few disease states. For maximum *in vivo* effectiveness, theoretically, an *ex vivo*- or *in vivo*-generated, activated T cell population should be in a state that can maximally orchestrate an immune response to cancer, infectious disease, or other disease states. The present invention provides methods to generate an increased number of more highly activated and more pure T cells that have surface

receptor and cytokine production characteristics that appear more healthy and natural than other expansion methods.

In addition, the present invention provides compositions of cell populations of any target cell, including T cell populations and parameters for producing the same, as well as providing other related advantages.

Poor hematological function puts patients at risk for infections, bleeding, and leads to other morbid conditions that increase patient care, hospitalization, etc. that can lead to morbidity and mortality. Specifically, patients whose neutrophil count falls below 500 per ul are at increased risk of infections. Additionally, patients with low platelet counts are at risk for bleeding, and often require platelet transfusions. Accelerating hematologic recovery reduces these risks.

Neupogen and Leukine are recombinant forms of cytokines G-CSF and GM-CSF that are used to restore neutrophil counts. However, they have limitations in that patients still become neutropenic. Granulocyte transfusions are also given but the neutrophils only survive for a few hours after their infusion. Low platelet counts may be treated with platelet transfusions to reduce the risk of severe bleeding. However, transfused platelets also survive only for hours. Further, patients may become allo-immunized after multiple transfusions, so that they fail to demonstrate an increase in platelet counts following transfusions. All transfusions are associated with some risk of blood borne pathogens, are costly, and limited by donor availability.

If reduced neutophil count (neutropenia) can be prevented or the number of days that patients experience this can be minimized, then the risk of infection can be reduced. This in turn should reduce patient care costs, use of antibiotics, and number of hospital days. Again, reduction of the number of days of low platelet counts should reduce patient care costs, risk of bleeding, and requirement for platelet transfusions.

Accordingly, there is a need in the art for methods of accelerating hematological recovery. The present invention provides methods for accelerating hematological recovery and provides other related advantages.

BRIEF SUMMARY OF THE INVENTION

The present invention provides a method for accelerating hematologic recovery in a patient exhibiting reduced hematologic function, comprising, contacting a population of cells from the patient wherein at least a portion thereof comprises T cells with a surface, wherein said surface has attached thereto a first agent that ligates a first cell surface moiety of a T cell, and the same or a second surface has attached thereto a second agent that ligates a second moiety of said T cell, wherein said ligation by the first and second agent induces proliferation of said T cell; administering to the patient the population of T cells generated as described herein, thereby accelerating hematologic recovery in the patient. In one embodiment, the first agent comprises an anti-CD3 antibody or an antigen binding fragment thereof and said second agent comprises a ligand which binds an accessory molecule on the surface of said T cells. In a further embodiment, the accessory molecule is CD28. In an additional embodiment, the first agent comprises an anti-CD3 antibody or an antigen binding fragment thereof and said second agent comprises an anti-CD28 antibody or an antigen binding fragment thereof. In further embodiments, the first and second agents are attached to the first or second surface by covalent, direct, or indirect attachment. In one embodiment, the patient is afflicted with a cancer. Cancers that may be treated with the invention described herein include, but are not limited to multiple myeloma, prostate cancer, and chronic lymphocytic leukemia. In one embodiment, the hematologic recovery can be any one or more of the following: an increase in neutrophil counts, an increase in platelet counts, an increase in hemoglobin levels, or an increase in NK cell counts.

Another aspect of the present invention provides a method for accelerating neutrophil recovery in a patient, comprising;contacting a population of cells from the patient wherein at least a portion thereof comprises T cells with a surface, wherein said surface has attached thereto a first agent that ligates a first cell surface moiety of a T cell, and the same or a second surface has attached thereto a second agent that ligates a second moiety of said T cell, wherein said ligation by the first and second agent induces proliferation of said T cell; administering to the patient the population of T cells of (i); thereby accelerating neutrophil cell recovery in the patient. In one

embodiment, the first agent comprises an anti-CD3 antibody or an antigen binding fragment thereof and said second agent comprises a ligand which binds an accessory molecule on the surface of said T cells. In a further embodiment, the accessory molecule is CD28. In an additonal embodiment, the first agent comprises an anti-CD3 antibody or an antigen binding fragment thereof and said second agent comprises an anti-CD28 antibody or an antigen binding fragment thereof. In one embodiment, the patient is afflicted with cancer. In this regard, the patient may be afflicted with multiple myeloma, prostate cancer, and chronic lymphocytic leukemia, and the like.

These and other aspects of the present invention will become evident upon reference to the following detailed description and attached drawings.

BRIEF DESCRIPTION OF THE SEVERAL VIEWS OF THE DRAWINGS

Figure 1 is a plot comparing the total numbers of activated and expanded T cells measured at day 8 starting with about 0.5 x 10^9 T cells with (XCELLERATE II™) or without (XCELLERATE I™) magnetic concentration and stimulation.

Figure 2 is a plot comparing fold expansion of activated and expanded T cells measured at day 8 with (XCELLERATE II™) or without (XCELLERATE I™) magnetic concentration and stimulation.

Figure 3 is a plot representing flow cytometry analysis of CD154 expression comparing restimulation of T cells previously cultured for 8 days after magnetic concentration and stimulation (XCELLERATE II™) or without magnetic concentration and stimulation (XCELLERATE I™).

Figure 4 is a plot representing flow cytometry analysis of CD154 expression following 3 days in culture comparing magnetic concentration and stimulation (XCELLERATE II™) with cells activated without magnetic concentration and stimulation (XCELLERATE I™).

Figures 5A-5B are plots depicting T cell activation and expansion with XCELLERATE I™ PBMC (5A) or PBMC having been frozen and thawed (5B) to initiate the XCELLERATE I™ process.

Figures 6A-6B are plots depicting time course analysis of CD25 expression following activation of T cells in one donor sample (PC071) during the XCELLERATE I or II™ process. Restimulation was performed at the 8 day mark to simulate *in vivo* activation. Figure 6A, depicts CD25 expression on $CD4^+$ cells, while Figure 6B depicts CD25 expression on $CD8^+$ cells.

Figures 7A-7B are plots depicting time course analysis of CD154 expression following activation of T cells in one donor sample (PC071) during the XCELLERATE I or II™ process. Restimulation was performed at the 8 day mark to simulate *in vivo* activation. Figure 7A, depicts CD154 expression on $CD4^+$ cells, while Figure 7B depicts CD154 expression on $CD8^+$ cells.

Figures 8A and 8B are plots illustrating growth of human peripheral blood T cells following stimulation with anti-CD3 and anti-CD28 co-immobilized beads utilizing process set forth in Example IX.

Figure 9 is a plot illustrating growth of human peripheral blood T cells following stimulation with anti-CD3 and anti-CD28 co-immobilized beads +/- recombinant human IL-2 at 10 u/ml and +/- monocyte depletion. All cells were cultured in Baxter Lifecell Flasks (300ml). Scale up refers to a 300ml flask culture (No IL-2/Monocyte depleted) that was expanded up to a Baxter Lifecell 3 Liter flask.

Figure 10 is a plot demonstrating the kinetic analysis of cell size as determined by forward scatter flow cytometry profiles over time.

Figures 11A and 11B are plots representing CD25 expression over time following initial stimulation with anti-CD3 and anti-CD28 co-immobilized beads. Figure 11A represents the expression profile of CD25 on $CD4^+$ cells, while Figure 11B represents the expression profile of CD25 on $CD8^+$ cells.

Figure 12 is a plot illustrates changes in cell size as determined by forward scatter flow cytometry profiles over time following primary and secondary stimulation.

Figures 13A and 13B are plots representing CD25 expression over time following primary and secondary stimulation. Figure 13A represents the expression

profile of CD25 on $CD4^+$ cells, while Figure 13B represents the expression profile of CD25 on $CD8^+$ cells.

Figures 14A and 14B are flow cytometry data plots representing CD154 expression following secondary stimulation, wherein primary and secondary stimulation sources were varied. Figure 14A represents the expression profile of CD154 on $CD4^+$ cells, while Figure 14B represents the expression profile of CD154 on $CD8^+$ cells.

Figure 15 is a flow cytometry data plot representing CD137 expression on all expanded T cells in sample following secondary stimulation.

Figures 16A and 16B are flow cytometry data plots representing CD54 expression following secondary stimulation, wherein secondary stimulation sources were varied. Figure 16A represents the expression of CD54 on $CD4^+$ cells, while Figure 16B represents the expression of CD54 on $CD8^+$ cells.

Figures 17A-17D are flow cytometry data plots representing cell phenotypes as well as CD154 and CD137 expression following secondary stimulation by anti-CD3 and anti-CD28 coupled beads of T cells obtained from a patient with B-cell chronic lymphocytic leukemia. Figures 17A and 17B represent $CD4^+$ and $CD8^+$ cells present in samples 13 days post-stimulation with anti-CD3 and anti-CD28 coupled beads (17A) and 18 days post-primary stimulation and 7 days post-secondary stimulation with anti-CD3 and anti-CD28 coupled beads (17B). Figures 17C and 17D are flow cytometry data plots representing CD154 and CD137 expression after secondary stimulation of cells obtained from a patient with B-cell chronic lymphocytic leukemia.

Figures 18A-18C are plots representing the expression over time of IL-2 (18A), Interferon gamma (IFN-γ) (18B), and IL-4 (18C) following primary and secondary stimulation of T cells from normal donors.

Figures 19A-19B are plots representing expression over time of CD62L following stimulation with anti-CD3 and anti-CD28 coupled beads.

Figure 20 is a plot depicting the percentage of CD4 or CD8 cells following stimulation with anti-CD3 and anti-CD28 co-immobilized beads.

Figures 21A-21B are plots representing flow cytometry data as a function of mean fluorescence intensity of CD25 and CD154 expression, respectively following stimulation with anti-CD3 and anti-CD28 co-immobilized beads and +/- re-stimulation utilizing process in Example IX.

Figures 22A-22B are plots representing flow cytometry analyses of CD154 staining versus control staining (*e.g.*, background) in cells with both CD4 and CD8 sub-populations (22A) or CD4-enriched populations (22B), prior to anti-CD3 and anti-CD28 co-immobilized bead stimulation.

Figures 23A-23B are plots representing ELISA analysis of TNF-α (23A) and IFN-γ (23B) in media following stimulation of peripheral blood lymphocytes with anti-CD3 and anti-CD28 co-immobilized beads.

Figures 24A-24B are plots representing ELISA analysis of IL-4 (24A) and IL-2 (24B) in media following stimulation of peripheral blood lymphocytes with anti-CD3 and anti-CD28 co-immobilized beads.

Figure 25 is a plot depicting increase in T cell size following stimulation of peripheral blood lymphocytes with anti-CD3 and anti-CD28 co-immobilized beads and using forward scatter analysis.

Figures 26A-26L are bar graphs representing flow cytometry data of CD62L expression (mean fluorescence intensity, MFI) (26A), CD49d (MFI) (26B), CD25 (MFI) (26C), CD69 (MFI) (26D), CD154 (MFI) (26E), forward light scatter (size) (26F), viability (% live gate) (26G); all following stimulation with anti-CD3 and anti-CD28 co-immobilized beads and re-stimulation with the same at day 8. Figures 26H-26L depict CD62L, CD69, CD49d, CD154, and CD25 at 4 and 18 hours post-stimulation, respectively.

Figure 27 is a graph depicting the fold increase of T cells over time following stimulation with anti-CD3 and anti-CD28 co-immobilized beads with varying ratios of CD3:CD28.

Figure 28 is a graph comparing expansion of T cells in a static system to expansion of T cells in the Wave Bioreactor.

Figure 29 is a graph comparing fold increase of polyclonal T cells to the fold increase of CMV pp65 A2-tetramer+ (antigen-specific) T cells using varying bead:cell ratios. Solid bars represent polyclonal T cells. Striped bars represent CMV-specific T cells.

Figures 30A-30C are graphs showing increases in neutrophils (30A), platelets (30B), and hemoglobin (30C) following infusion of Xcellerated T cells in CLL patients.

DETAILED DESCRIPTION OF THE INVENTION

Prior to setting forth the invention, it may be helpful to an understanding thereof to set forth definitions of certain terms that will be used hereinafter.

The term "biocompatible", as used herein, refers to the property of being predominantly non-toxic to living cells.

The term "stimulation", as used herein, refers to a primary response induced by ligation of a cell surface moiety. For example, in the context of receptors, such stimulation entails the ligation of a receptor and a subsequent signal transduction event. With respect to stimulation of a T cell, such stimulation refers to the ligation of a T cell surface moiety that in one embodiment subsequently induces a signal transduction event, such as binding the TCR/CD3 complex. Further, the stimulation event may activate a cell and upregulate or downregulate expression or secretion of a molecule, such as downregulation of TGF-ß. Thus, ligation of cell surface moieties, even in the absence of a direct signal transduction event, may result in the reorganization of cytoskeletal structures, or in the coalescing of cell surface moieties, each of which could serve to enhance, modify, or alter subsequent cell responses.

The term "activation", as used herein, refers to the state of a cell following sufficient cell surface moiety ligation to induce a noticeable biochemical or morphological change. Within the context of T cells, such activation, refers to the state of a T cell that has been sufficiently stimulated to induce cellular proliferation. Activation of a T cell may also induce cytokine production and performance of

regulatory or cytolytic effector functions. Within the context of other cells, this term infers either up or down regulation of a particular physico-chemical process.

The term "force", as used herein, refers to an artificial or external force applied to the cells to be stimulated that induces cellular concentration and concentration of cells with the agent that binds a cell surface moiety. For example, the term "force" includes any force greater than gravity (*i.e.*, in addition to gravity and not solely gravitational force) that induces cell concentration and/or cell surface moiety aggregation. Such forces include transmembrane pressure such as filtration, a hydraulic force, an electrical force, an acoustical force, a centrifugal force, or a magnetic force. Ideally, the force utilized drives the concentration of the target cell of interest with an agent that ligates a cell surface moiety. In various contexts, the force can be pulsed, *i.e.*, applied and reapplied (*e.g.*, a magnetic force could be turned off and on, pulsing the population of cells in combination with a paramagnetic particle).

The term "simultaneous", as used herein, refers to the fact that inherently upon concentrating cells at a surface that has cell surface moiety binding agents attached thereto, results in concentration of cells with each other and with the surface, thus ligands (*i.e.*, agents). However, the use of the term "simultaneous" does not preclude previous binding of the target cells with a surface having cell surface moiety binding agents attached thereto, as concentration and further ligand binding occurs simultaneously at the concentration surface. For example, within the context of T cell activation, the T cells may be exposed to a surface such as a paramagnetic bead having anti-CD3 and anti-CD28 antibodies attached thereto and subsequently concentrated by a magnetic field. Thus, in this context while cells and beads have previous contact and ligation, nevertheless, during concentration of cells additional ligation occurs.

The term "target cell", as used herein, refers to any cell that is intended to be stimulated by cell surface moiety ligation.

An "antibody", as used herein, includes both polyclonal and monoclonal antibodies; primatized (*e.g.*, humanized); murine; mouse-human; mouse-primate; and chimeric; and may be an intact molecule, a fragment thereof (such as scFv, Fv, Fd, Fab, Fab' and $F(ab)'_2$ fragments), or multimers or aggregates of intact molecules and/or

fragments; and may occur in nature or be produced, *e.g.*, by immunization, synthesis or genetic engineering; an "antibody fragment," as used herein, refers to fragments, derived from or related to an antibody, which bind antigen and which in some embodiments may be derivatized to exhibit structural features that facilitate clearance and uptake, *e.g.*, by the incorporation of galactose residues. This includes, *e.g.*, F(ab), $F(ab)'_2$, scFv, light chain variable region (V_L), heavy chain variable region (V_H), and combinations thereof.

The term "protein", as used herein, includes proteins, polypeptides and peptides; and may be an intact molecule, a fragment thereof, or multimers or aggregates of intact molecules and/or fragments; and may occur in nature or be produced, *e.g.*, by synthesis (including chemical and/or enzymatic) or genetic engineering.

The term "agent", "ligand", or "agent that binds a cell surface moiety", as used herein, refers to a molecule that binds to a defined population of cells. The agent may bind any cell surface moiety, such as a receptor, an antigenic determinant, or other binding site present on the target cell population. The agent may be a protein, peptide, antibody and antibody fragments thereof, fusion proteins, synthetic molecule, an organic molecule (*e.g.*, a small molecule), or the like. Within the specification and in the context of T cell stimulation, antibodies are used as a prototypical example of such an agent.

The terms "agent that binds a cell surface moiety" and "cell surface moiety", as used herein, are used in the context of a ligand/anti-ligand pair. Accordingly, these molecules should be viewed as a complementary/anti-complementary set of molecules that demonstrate specific binding, generally of relatively high affinity (an affinity constant, K_a, of about 10^6 M^{-1}).

A "co-stimulatory signal", as used herein, refers to a signal, which in combination with a primary signal, such as TCR/CD3 ligation, leads to T cell proliferation.

A "ligand/anti-ligand pair", as used herein, refers to a complementary/anti-complementary set of molecules that demonstrate specific binding, generally of relatively high affinity (an affinity constant, K_a, of about 10^6 M^{-1},).

Exemplary ligand/anti-ligand pairs enzyme/inhibitor, hapten/antibody, lectin/carbohydrate, ligand/receptor, and biotin/avidin or streptavidin. Within the context of the present invention specification receptors and other cell surface moieties are anti-ligands, while agents (*e.g.*, antibodies and antibody fragments) reactive therewith are considered ligands.

"Separation", as used herein, includes any means of substantially purifying one component from another (*e.g.*, by filtration or magnetic attraction).

"Quiescent", as used herein, refers to a cell state wherein the cell is not actively proliferating.

A "surface", as used herein, refers to any surface capable of having an agent attached thereto and includes, without limitation, metals, glass, plastics, co-polymers, colloids, lipids, cell surfaces, and the like. Essentially any surface that is capable of retaining an agent bound or attached thereto. A prototypical example of a surface used herein, is a particle such as a bead.

One aspect of the present invention is directed to the surprising finding that infusion of Xcellerated™ T cells as described herein accelerates hematologic recovery. For example, a Phase I/II Clinical Trial of patients with multiple myeloma was conducted in which patients received high dose myeloablative chemotherapy consisting of 200 milligrams per meter squared followed by an autologous peripheral blood stem cell transplant. After this regimen, patients are typically neutropenic (*i.e.*, have a neutrophil count below 500 per ul for about 8 days). In this clinical trial, patients were administered one infusion of Xcellerated T Cells on day 3 after the transplant. Of the first 20 patients that received the transplant and the infusion of Xcellerated T cells, the overall patient average is about 3 days of neutropenia. Several patients appear never to have had any neutropenic period at all, which is highly unusual if not unheard of (for example, an analysis of historical data in a series of 142 patients receiving the same treatment/transplant, the least number of days of neutropenia in those 142 patients was four days). These preliminary data indicate that the Xcellerated™ T cells accelerate not only neutrophil recovery but also recovery of platelets and possibly red blood cells. Thus, the present invention provides methods for

accelerating hematologic recovery. Any increase in recovery rates beyond rates typically seen in the relevant disease setting (*e.g.*, post-transplant) is understood as accelerating hematologic recovery.

The primary measure of hematologic recovery is neutrophil count. Neutrophils usually constitute about 45 to 75% of all white blood cells in the bloodstream. When the neutrophil count falls below 1,000 cells per microliter of blood, the risk of infection increases somewhat; when it falls below 500 cells per microliter, the risk of infection increases greatly. Without the key defense provided by neutrophils, controlling infections is problematic and patients are at risk of dying from an infection. Accordingly, in clinical settings, such as transplant settings, the sooner neutrophil counts recover, the sooner a patient can be released from the hospital. Accordingly, any decrease in time that it takes to achieve clinically relevant levels of neutrophils is beneficial to the patient and contemplated herein as acceleration of hematologic recovery.

Hematologic recovery can also be measured by a clinically relevant recovery of platelets (as would be recognized by the skilled artisan, there are normally between 150,000-450,000 platelets in each microliter of blood) and a clinically relevant recovery of hemoglobin levels. Thus, any increase in the rapidity of a clinically relevant recovery of platelets and hemoglobin levels (Hemoglobin levels (varies with altitude) are generally as follows: Male: 13.8 to 17.2 gm/dl Female: 12.1 to 15.1 gm/dl) is advantageous and contemplated herein. In certain embodiments, rapidity in NK cell recovery is also an indicator of accelerated hematologic recovery.

An additional aspect of the present invention is directed to the surprising finding that the combination of a force which induces the concentration of cells, ligation of cell surface moieties, and culturing cells in a rocking, closed system, results in a profound enhancement in activation and expansion of these cells. In the prototypic example set forth herein, T cells are utilized. However, one of skill in the art would readily conclude that the present invention has broad applicability to any cell type where cell surface moiety ligation or aggregation is desired or where such binding leads to a subsequent cellular signaling event (*e.g.*, receptors). While not wishing to be

bound by theory, certain embodiments of the present invention may function by taking advantage of a phenomenon involving lipid rafting and/or receptor polarization. The phenomena are similar in that they suggest either initiation/enhancement of signal transduction by the aggregation of lipid rafts comprising cell surface moieties or enhanced signal transduction due to localization (*i.e.*, polarization) of receptors at one, or even several area(s) of a cell. Thus, not only does such cell surface moiety ligation lead to unexpectedly robust cell activation and proliferation in T cells but can also be applied to magnifying the signal transduction event of many cell types. Additionally, while still not wishing to be bound by theory, certain aspects of the present invention may function by providing optimal aeration for the expanding cells. Thus, cell surface moiety ligation combined with aeration through rocking and perfused media lead to unexpectedly robust cell activation and expansion of T cells to unexpectedly high densities and absolute numbers. Accordingly, within the context of T cells, the present invention provides a variety of unexpected advantages, first it eliminates the need for a separate monocyte-depletion step using "uncoated" particles, simplifies expansion of T cells by requiring fewer cell transfers and fewer reagents, increased level of T cell activation during activation process, significantly reduces the time to achieve cell numbers adequate for cell therapy, reduces time and labor involved in the processing of the cells, reduces the cost of manufacturing, and increases the flexibility of scheduling patient processing and infusions.

In an additional aspect of the present invention, a first and second or more surfaces are utilized with or without ligands/agents bound thereto. In this embodiment, the various surfaces may have the same or different agents attached thereto for binding cell surface moieties of target cells. For example, a paramagnetic bead may have attached thereto an antibody for a receptor on a target cell and such beads may be mixed with a population of cells containing the target cell. Further, the cell population may be mixed with a second or more bead with the same or different cell surface moiety binding agents attached thereto. In certain embodiments, force induced concentration is carried out, upon which the beads and cells are brought together in a smaller volume and thus signaling is magnified. In another example,

paramagnetic beads that have an agent specific for a carbohydrate or other non-receptor cell surface moiety attached thereto are mixed with a population of cells containing the target cell. A magnetic field is then used to draw the bead attached cells to another surface that has receptor ligating agents attached thereto. Thus, the signal transduction inducing agent is on the second surface. In yet another example, an agent that binds a cell surface moiety of target cell may be attached to a particle large enough to be retained in a mesh or filter that itself may have ligands attached thereto.

As noted above, the present invention provides methods for stimulating a cell population by binding moieties on the surfaces of the cells in that population. Contacting a cell population with an agent (*e.g.*, a ligand) that binds to a cell surface moiety can stimulate the cell population. The ligand may be in solution but also may be attached to a surface. Ligation of cell surface moieties, such as a receptor, may generally induce a particular signaling pathway. Recent studies suggest that for signaling to occur, critical concentrations of lipid rafts containing the requisite receptors must aggregate. By way of example, raft aggregation may be facilitated *in vivo* or *in vitro* by attaching ligands for particular cell surface moieties to paramagnetic particles, exposing the ligand-bearing particles to the cells, and shortly thereafter or simultaneously applying a force, such as a magnetic field to assist polarizing the ligated moieties (*e.g.,* receptors) and concentrating cells in a small volume. The application of a magnetic force concentrates the cells as well as concentrating the cells with the surface having agents attached thereto that ligate cell surface moieties, thereby bringing greater contact of the cells with the ligands, resulting in accelerated and more potent activation. Many applications of the present invention are possible, for example, if cells have low numbers of and/or dysfunctional receptors, the method may sufficiently concentrate such receptors in the lipid rafts to overcome such defects and to permit proper signaling activity. One example of such cell surface repertoire correction is in patients with certain types of leukemia, wherein prior to cell surface moiety stimulation with agents such as anti-CD3 and anti-CD28 antibodies several normal cell surface markers are unusually low, such as the CD3/TCR complex. By stimulating these cell populations with agents such as anti-CD3 and anti-CD28 antibodies, the cell surface

markers of these cells return to a level that appears normal and as such can provide a more robust immunotherapy product for cancer therapy that provides a stronger and more rapid immune response when returned to the patient. In yet other applications of this invention, cells may be efficiently concentrated and activated, including inducing receptor polarization, thereby maximizing receptor signaling events. Such applications have broad utility including the use in screening assays directed at receptors or by collecting cellular rafts on the surface of a cell to induce activation such as inducing apoptosis by ligating Fas or like molecules in a tumor cell.

In one example of such screening assays, one could use G-coupled protein receptor bearing cells and contact them with agents that bind thereto, these agents being bound to a surface that allows force induced concentration. Accordingly, as the receptors raft together the signal transduction event would be amplified. This could be important in the study of signal transduction events that are very low level in typical experiments and thus screening for drug compounds to inhibit or somehow modify such signal transduction events.

Stimulation Of A Cell Population

The methods of the present invention relate to the stimulation of a target cell by introducing a ligand or agent that binds to a cellular moiety, inducing a cellular event. Binding of the ligand or agent to the cell may trigger a signaling pathway that in turn activates particular phenotypic or biological changes in the cell. The stimulation of a target cell by introducing a ligand or agent that binds to a cellular moiety as described herein may upregulate or downregulate any number of cellular processes leading to particular phenotypic or biological changes in the cell. The activation of the cell may enhance normal cellular functions or initiate normal cell functions in an abnormal cell. Certain methods described herein provide stimulation by forcing concentration of the cells together with the ligand or agent that binds a cell surface moiety. Stimulation of a cell may be enhanced or a particular cellular event may be stimulated by introducing a second agent or ligand that ligates a second cell surface moiety. This method may be applied to any cell for which ligation of a cell surface moiety leads to a signaling event.

The invention further provides means for selection or culturing the stimulated cells. The prototypic example described is stimulation of T cells, but one of ordinary skill in the art will readily appreciate that the method may be applied to other cell types. By way of example, cell types that may be stimulated and selected include fibroblasts, neuroblasts, lung cells, hematologic stem cells and hematopoietic progenitor cells ($CD34^+$ cells), mesenchymal stem cells, mesenchymal progenitor cells, neural and hepatic progenitor and stem cells, dendritic cells, cytolytic T cells ($CD8^+$ cells), B-cells, NK cells, other leukocyte populations, pluripotent stem cells, multi-potent stem cells, islet cells, etc. Accordingly, the present invention also provides populations of cells resulting from this methodology as well as cell populations having distinct phenotypical characteristics, including T cells with specific phenotypic characteristics.

As noted above a variety of cell types may be utilized within the context of the present invention. For example, cell types such as B cells, T cells, NK cells, other blood cells, neuronal cells, lung cells, glandular (endocrine) cells, bone forming cells (osteoclasts, etc.), germ cells (*e.g.,* oocytes), epithelial cells lining reproductive organs, and others may be utilized. Cell surface moiety-ligand pairs could include (but not exclusively): T cell antigen receptor (TCR) and anti-CD3 mAb, TCR and major histocompatibility complex (MHC)+antigen, TCR and peptide-MHC tetramer, TCR and superantigens (*e.g.*, staphylococcal enterotoxin B (SEB), toxic shock syndrome toxin (TSST), *etc.*), B cell antigen receptor (BCR) and anti-Ig, BCR and LPS, BCR and specific antigens (univalent or polyvalent), NK receptor and anti-NK receptor antibodies, FAS (CD95) receptor and FAS ligand, FAS receptor and anti-FAS antibodies, CD54 and anti-CD54 antibodies, CD2 and anti-CD2 antibodies, CD2 and LFA-3 (lymphocyte function related antigen-3), cytokine receptors and their respective cytokines, cytokine receptors and anti-cytokine receptor antibodies, TNF-R (tumor necrosis factor-receptor) family members and antibodies directed against them, TNF-R family members and their respective ligands, adhesion/homing receptors and their ligands, adhesion/homing receptors and antibodies against them, oocyte or fertilized oocyte receptors and their ligands, oocyte or fertilized oocyte receptors and antibodies against them, receptors on the endometrial lining of uterus and their ligands, hormone

receptors and their respective hormone, hormone receptors and antibodies directed against them, and others.

The nature of the binding of a receptor by a ligand will either result in the multimerization of the receptors, or aggregation/orientation of the receptors, such that signaling or cell response is upregulated, downregulated, accelerated, improved, or otherwise altered so as to confer a particular benefit, such as cell division, cytokine secretion, cell migration, increased cell-cell interaction, etc.

Two examples are given below that illustrate how such a multimerization, aggregation, or controlled reorientation of cell surface moieties could be of practical benefit.

In one example, normal T cell activation by antigen and antigen presenting cells usually results in aggregation of TCR rafts, cytoskeletal reorganization, polarization of "activation" signals and cell division, for example. Using man-made approaches, such as those described herein, in the absence of "normal" *in-vivo* T cell activation, one could accelerate, improve, or otherwise affect the functions described above, in particular through the accelerated, controlled, and spatially oriented ligation of TCR and CD28. Benefits could be improved cell expansion *in vitro* resulting in higher numbers of infuseable and more robust cells for therapeutic applications. In particular, the present invention provides for methods of activating and expanding T cells to very high densities (ranging from 6 X 10^6 cells/ml to 90 X 10^6 cells/ml) and results in production of very high number of cells (as many as 800 billion cells are expanded from one individual from a starting number of cells of about 0.5 X 10^9 cells) Other benefits could be improved receptor "aggregation" for cells with defects, such as lower-than-normal TCR density on the cell surface. Similarly, *in vivo* applications could be beneficial where specific T cell populations need to be activated, such as tumor-specific T cells at tumor sites. Improved receptor aggregation and orientation could provide an activation signal otherwise difficult to obtain for functionally tolerized T cells. Further, such activation could be used within the context of antigen specific T cells. In this regard T cells from a tumor could be isolated and expanded and infused

into the patient. Similarly, T cells exposed to an antigen either *in vivo* or *in vitro* could be expanded by the present methodologies.

In another example, improved induction of cell death occurs via the FAS pathway: The ability to accelerate the multimerization of FAS, spatially orient "activated" FAS on target cell surfaces, or to promote a cumulative FAS ligation that would otherwise be unachievable, could provide significant benefit *in vivo*, particularly for treating cancer, autoimmune responses, or graft-versus-host disease. For example, a tumor cell may express low levels of FAS *in vivo*, and the host may express low levels of FAS-L at tumor sites (due to suppressive cytokines, *etc.*). Due to these low levels, an adequate FAS signal cannot be generated, allowing for tumor survival and growth. One possible way to overcome this FAS/FAS-ligand deficiency could be to target tumors/tumor sites with monovalent or multivalent ligands for FAS (FAS-L, antibodies, etc.), bound to paramagnetic particles. Application of a strong magnetic field using the present at tumor sites (*e.g.*, melanoma, Kaposi's sarcoma, squamous cell neck carcinomas, etc.) could provide for the spatial orientation of the paramagnetic particles at tumor sites as the particles bound FAS on tumor cells, adapted for receptor activation and/or T cell activation and expansion. Increased FAS aggregation accompanied by signal polarization might provide adequate signal to now induce cell death in the tumor cells.

In one embodiment of the invention, a T cell population may be stimulated by simultaneously concentrating and ligating the surfaces of the T cells. In one aspect of the present invention, antibodies to CD3 and CD28 are co-immobilized on a surface. In one embodiment, the surface for such immobilization includes particles, and in certain aspects, beads, such as paramagnetic beads. In another aspect of the present invention, any ligand that binds the TCR/CD3 complex and initiates a primary stimulation signal may be utilized as a primary activation agent immobilized on the surface. Any ligand that binds CD28 and initiates the CD28 signal transduction pathway, thus causing co-stimulation of the cell with a CD3 ligand and enhancing activation of a population of T cells, is a CD28 ligand and accordingly, is a co-stimulatory agent within the context of the present invention. In a further aspect of the

invention, a force is applied to the mixture of T cells and anti-CD3 and anti-CD28-conjugated surfaces to concentrate the T cells, thus maximizing T cell surface ligation. While in one particular embodiment the concentration force is magnetic force applied where the anti-CD3 and anti-CD28 coated surfaces are paramagnetic beads, other means to bring the cells and the ligands together in a concentrated fashion are available in the art. Such methods of stimulating a T cell population provides significant bead-cell and/or cell-cell contact that induces surprisingly greater activation and/or proliferation of T cells. Furthermore, the inventive methods alter the cell surface marker profile wherein the activated T cells express cell surface markers that indicate a more normal phenotype and less variable final product compared to the profile of the T cells when first isolated from a subject with a disease.

The Primary Signal

The biochemical events responsible for *ex vivo* T cell stimulation are set forth briefly below. Interaction between the TCR/CD3 complex and antigen presented in conjunction with either MHC class I or class II molecules on an antigen-presenting cell initiates a series of biochemical events termed antigen-specific T cell activation. Accordingly, activation of T cells can be accomplished by stimulating the T cell TCR/CD3 complex via direct stimulation of the TCR or CD3, or by stimulating the CD2 surface protein. An anti-CD3 monoclonal antibody can be used to activate a population of T cells via the TCR/CD3 complex. A number of anti-human CD3 monoclonal antibodies are commercially available, exemplary are OKT3, prepared from hybridoma cells obtained from the American Type Culture Collection, and monoclonal antibody G19-4. Similarly, stimulatory forms of anti-CD2 antibodies are known and available. Stimulation through CD2 with anti-CD2 antibodies is typically accomplished using a combination of at least two different anti-CD2 antibodies. Stimulatory combinations of anti-CD2 antibodies that have been described include the following: the T11.3 antibody in combination with the T11.1 or T11.2 antibody (Meuer *et al.*, *Cell 36*:897-906, 1984), and the 9.6 antibody (which recognizes the same epitope as T11.1) in combination with the 9-1 antibody (Yang *et al.*, *J. Immunol. 137*:1097-

1100, 1986). Other antibodies that bind to the same epitopes as any of the above described antibodies can also be used. Additional antibodies, or combinations of antibodies, can be prepared and identified by standard techniques.

A primary activation signal can also be delivered to a T cell through other mechanisms. For example, a combination that may be used includes a protein kinase C (PKC) activator, such as a phorbol ester (*e.g.*, phorbol myristate acetate), and a calcium ionophore (*e.g.*, ionomycin, which raises cytoplasmic calcium concentrations), or the like. The use of such agents bypasses the TCR/CD3 complex but delivers a stimulatory signal to T cells. Other agents acting as primary signals may include natural and synthetic ligands. A natural ligand may include MHC with or without a peptide presented. Other ligands may include, but are not limited to, a peptide, polypeptide, growth factor, cytokine, chemokine, glycopeptide, soluble receptor, steroid, hormone, mitogen, such as PHA, or other superantigens, peptide-MHC tetramers (Altman, et al., Science. 1996 Oct 4;274(5284):94-6.) and soluble MHC dimers (Dal Porto, et al. *Proc Natl Acad Sci U S A* 1993 Jul 15;90). . Within the context of the present invention, the use of concentration and stimulation may result in such high receptor polarization that no secondary signal is required to induce proliferation of T cells.

In other embodiments, signal transduction events of any kind may be magnified or analyzed by utilizing the current invention. For example, G protein-coupled receptors may stimulated and measured using the concentration methods of the present invention.

The Secondary Signal

While stimulation of the TCR/CD3 complex or CD2 molecule appears to be required for delivery of a primary activation signal in a T cell, a number of molecules on the surface of T cells, termed accessory or co-stimulatory molecules, have been implicated in regulating the transition of a resting T cell to blast transformation, and subsequent proliferation and differentiation. Thus, in addition to the primary activation signal, induction of T cell responses requires a second, co-stimulatory signal.

One such co-stimulatory or accessory molecule, CD28, is believed to initiate or regulate a signal transduction pathway that is distinct from any stimulated by the TCR complex.

Therefore, to enhance activation and proliferation of a population of T cells in the absence of exogenous growth factors or accessory cells, an accessory molecule on the surface of the T cell, such as CD28, is stimulated with a ligand that binds the accessory molecule. In one embodiment, stimulation of the accessory molecule CD28 and T cell activation occur simultaneously by contacting a population of T cells with a surface to which a ligand that binds CD3 and a ligand that binds CD28 are attached. Activation of the T cells, for example, with an anti-CD3 antibody, and stimulation of the CD28 accessory molecule results in selective proliferation of $CD4^+$ T cells.

Accordingly, one of ordinary skill in the art will recognize that any agent, including an anti-CD28 antibody or fragment thereof capable of cross-linking the CD28 molecule, or a natural ligand for CD28 can be used to stimulate T cells. Exemplary anti-CD28 antibodies or fragments thereof useful in the context of the present invention include monoclonal antibody 9.3 ($IgG2_a$) (Bristol-Myers Squibb, Princeton, NJ), monoclonal antibody KOLT-2 (IgG1), 15E8 (IgG1), 248.23.2 (IgM), and EX5.3D10 ($IgG2_a$) (ATCC HB11373). Exemplary natural ligands include the B7 family of proteins, such as B7-1 (CD80) and B7-2 (CD86) (Freedman *et al.*, *J. Immunol. 137*:3260-3267, 1987; Freeman *et al.*, *J. Immunol. 143*:2714-2722, 1989; Freeman *et al.*, *J. Exp. Med. 174*:625-631, 1991; Freeman *et al.*, *Science 262*:909-911, 1993; Azuma *et al.*, *Nature 366*:76-79, 1993; Freeman *et al.*, *J. Exp. Med. 178*:2185-2192, 1993). In addition, binding homologues of a natural ligand, whether native or synthesized by chemical or recombinant techniques, can also be used in accordance with the present invention. Other agents acting as secondary signals may include natural and synthetic ligands. Agents may include, but are not limited to, other antibodies or fragments thereof, a peptide, polypeptide, growth factor, cytokine, chemokine, glycopeptide, soluble receptor, steroid, hormone, mitogen, such as PHA, or other superantigens.

In a further embodiment of the invention, activation of a T cell population may be enhanced by co-stimulation of other T cell integral membrane proteins. For example, binding of the T cell integrin LFA-1 to its natural ligand, ICAM-1, may enhance activation of cells. Another cell surface molecule that may act as a co-stimulator for T cells is VCAM-1 (CD106) that binds very-late-antigen-4 (VLA-4) on T cells. Ligation of 4-1BB, a co-stimulatory receptor expressed on activated T cells, may also be useful in the context of the present invention to amplify T cell mediated immunity.

One of skill in the art will appreciate that cells other than T cells may be stimulated by binding of an agent that ligates a cell surface moiety and induces aggregation of the moiety, which in turn results in activation of a signaling pathway. Other such cell surface moieties include, but are not limited to, GPI-anchored folate receptor (CD59), human IgE receptor (FcεRi receptor), BCR, EGF receptor, insulin receptor, ephrin B1 receptor, neurotrophin, glial-cell derived neutrophic factor (GNDF), hedgehog and other cholesterol-linked and palmitoylated proteins, H-Ras, integrins, endothelial nitric oxide synthase (eNOS), FAS, members of the TNF receptor family, GPI-anchored proteins, doubly acylated proteins, such as the Src-family kinases, the alpha-subunit of heterotrimeric G proteins, and cytoskeletal proteins.

Expansion Of T cell Population

In one aspect of the present invention, *ex vivo* T cell expansion can be performed by isolation of T cells and subsequent stimulation. In one embodiment of the invention, the T cells may be stimulated by a single agent. In another embodiment, T cells are stimulated with two agents, one that induces a primary signal and a second that is a co-stimulatory signal. Ligands useful for stimulating a single signal or stimulating a primary signal and an accessory molecule that stimulates a second signal may be used in soluble form, attached to the surface of a cell, or immobilized on a surface as described herein. A ligand or agent that is attached to a surface serves as a "surrogate" antigen presenting cell (APC). In one embodiment both primary and secondary agents are co-immobilized on a surface. In one embodiment, the molecule

providing the primary activation signal, such as a CD3 ligand, and the co-stimulatory molecule, such as a CD28 ligand, are coupled to the same surface, for example, a particle. Further, as noted earlier, one, two, or more stimulatory molecules may be used on the same or differing surfaces.

Prior to expansion, a source of T cells is obtained from a subject. The term "subject" is intended to include living organisms in which an immune response can be elicited (*e.g.*, mammals). Examples of subjects include humans, dogs, cats, mice, rats, and transgenic species thereof. T cells can be obtained from a number of sources, including peripheral blood mononuclear cells, bone marrow, lymph node tissue, spleen tissue, and tumors. In certain embodiments of the present invention, any number of T cell lines available in the art, may be used. In certain embodiments of the present invention, T cells can be obtained from a unit of blood collected from a subject using any number of techniques known to the skilled artisan, such as ficoll separation. In one embodiment, cells from the circulating blood of an individual are obtained by apheresis or leukapheresis. The apheresis product typically contains lymphocytes, including T cells, monocytes, granulocytes, B cells, other nucleated white blood cells, red blood cells, and platelets. In one embodiment, the cells collected by apheresis may be washed to remove the plasma fraction and to place the cells in an appropriate buffer or media for subsequent processing steps. In one embodiment of the invention, the cells are washed with phosphate buffered saline (PBS). In an alternative embodiment, the wash solution lacks calcium and may lack magnesium or may lack many if not all divalent cations. Again, surprisingly, initial activation steps in the absence of calcium lead to magnified activation. As those of ordinary skill in the art would readily appreciate a washing step may be accomplished by methods known to those in the art, such as by using a semi-automated "flow-through" centrifuge (for example, the Cobe 2991 cell processor) according to the manufacturer's instructions. After washing, the cells may be resuspended in a variety of biocompatible buffers, such as, for example, Ca-free, Mg-free PBS. Alternatively, the undesirable components of the apheresis sample may be removed and the cells directly resuspended in culture media.

In another embodiment, T cells are isolated from peripheral blood lymphocytes by lysing the red blood cells and depleting the monocytes, for example, by centrifugation through a PERCOLL™ gradient. A specific subpopulation of T cells, such as $CD28^+$, $CD4^+$, $CD8^+$, $CD45RA^+$, and $CD45RO^+$ T cells, can be further isolated by positive or negative selection techniques. For example, in one embodiment, T-cells are isolated by incubation with anti-CD3/anti-CD28 (*i.e.*, 3x28)-conjugated beads, such as DYNABEADS® M-450 CD3/CD28 T, for a time period sufficient for positive selection of the desired T cells. In one embodiment, the time period is about 30 minutes. In a further embodiment, the time period ranges from 30 minutes to 36 hours or longer and all integer values there between. In a further embodiment, the time period is at least 1, 2, 3, 4, 5, or 6 hours. In yet another embodiment, the time period is 10 to 24 hours. In one embodiment, the incubation time period is 24 hours. For isolation of T cells from patients with leukemia, use of longer incubation times, such as 24 hours, can increase cell yield. Longer incubation times may be used to isolate T cells in any situation where there are few T cells as compared to other cell types, such in isolating tumor infiltrating lymphocytes (TIL) from tumor tissue or from immunocompromised individuals. Further, use of longer incubation times can increase the efficiency of capture of CD8+ T cells.

Enrichment of a T-cell population by negative selection can be accomplished with a combination of antibodies directed to surface markers unique to the negatively selected cells. In one embodiment, the method is cell sorting and/or selection via negative magnetic immunoadherence or flow cytometry that uses a cocktail of monoclonal antibodies directed to cell surface markers present on the cells negatively selected. For example, to enrich for $CD4^+$ cells by negative selection, a monoclonal antibody cocktail typically includes antibodies to CD14, CD20, CD11b, CD16, HLA-DR, and CD8.

For isolation of a desired population of cells by positive or negative selection, the concentration of cells and surface (*e.g.* particles such as beads) can be varied. In certain embodiments, it may be desirable to significantly decrease the volume in which beads and cells are mixed together (*i.e.*, increase the concentration of

cells), to ensure maximum contact of cells and beads. For example, in one embodiment, a concentration of 2 billion cells/ml is used. In one embodiment, a concentration of 1 billion cells/ml is used. In a further embodiment, greater than 100 million cells/ml is used. In a further embodiment, a concentration of cells of 10, 15, 20, 25, 30, 35, 40, 45, or 50 million cells/ml is used. In yet another embodiment, a concentration of cells from 75, 80, 85, 90, 95, or 100 million cells/ml is used. In further embodiments, concentrations of 125 or 150 million cells/ml can be used. Using high concentrations can result in increased cell yield, cell activation, and cell expansion. Further, use of high cell concentrations allows more efficient capture of cells that may weakly express target antigens of interest, such as CD28-negative T cells, or from samples where there are many tumor cells present (*i.e.*, leukemic blood, tumor tissue, *etc*). Such populations of cells may have therapeutic value and would be desirable to obtain. For example, using high concentration of cells allows more efficient selection of CD8+ T cells that normally have weaker CD28 expression.

In a related embodiment, it may be desirable to use lower concentrations of cells. By significantly diluting the mixture of T cells and surface (*e.g.* particles such as beads), interactions between the particles and cells is minimized. This selects for cells that express high amounts of desired antigens to be bound to the particles. For example, CD4+ T cells express higher levels of CD28 and are more efficiently captured than CD8+ T cells in dilute concentrations. In one embodiment, the concentration of cells used is 5 X 10^6/ml. In other embodiments, the concentration used can be from about 1 X 10^5/ml to 1 X 10^6/ml, and any integer value in between.

If desired or necessary, monocyte populations (*i.e.*, $CD14^+$ cells) may be depleted from blood preparations prior to *ex vivo* expansion by a variety of methodologies, including anti-CD14 coated beads or columns, or utilization of the phagocytotic activity of these cells to facilitate removal. Accordingly, in one embodiment, the invention uses paramagnetic particles of a size sufficient to be engulfed by phagocytotic monocytes. In certain embodiments, the paramagnetic particles are commercially available beads, for example, those produced by Dynal AS under the trade name Dynabeads™. Exemplary Dynabeads™ in this regard are M-280,

M-450, and M-500. In one aspect, other non-specific cells are removed by coating the paramagnetic particles with "irrelevant" proteins (*e.g.*, serum proteins or antibodies). Irrelevant proteins and antibodies include those proteins and antibodies or fragments thereof that do not specifically target the T-cells to be expanded. In certain embodiments the irrelevant beads include beads coated with sheep anti-mouse antibodies, goat anti-mouse antibodies, and human serum albumin.

In brief, such depletion of monocytes is performed by preincubating PBMC isolated from whole blood or apheresed peripheral blood with one or more varieties of irrelevant or non-antibody coupled paramagnetic particles at any amount that allows for removal of monocytes (approximately a 20:1 bead:cell ratio) for about 30 minutes to 2 hours at 22 to 37 degrees C, followed by magnetic removal of cells which have attached to or engulfed the paramagnetic particles. Such separation can be performed using standard methods available in the art. For example, any magnetic separation methodology may be used including a variety of which are commercially available, (*e.g.*, DYNAL® Magnetic Particle Concentrator (DYNAL MPC®)). Assurance of requisite depletion can be monitored by a variety of methodologies known to those of ordinary skill in the art, including flow cytometric analysis of CD14 positive cells, before and after said depletion.

T cells for stimulation can also be frozen after the washing step, which does not require the monocyte-removal step. Wishing not to be bound by theory, the freeze and subsequent thaw step provides a more uniform product by removing granulocytes and to some extent monocytes in the cell population. After the washing step that removes plasma and platelets, the cells may be suspended in a freezing solution. While many freezing solutions and parameters are known in the art and will be useful in this context, one method involves using PBS containing 20% DMSO and 8% human serum albumin, or other suitable cell freezing media, the cells then are frozen to -80°C at a rate of 1° per minute and stored in the vapor phase of a liquid nitrogen storage tank. Other methods of controlled freezing may be used as well as uncontrolled freezing immediately at –20° C. or in liquid nitrogen.

The cell population may be stimulated as described herein, such as by contact with an anti-CD3 antibody or an anti-CD2 antibody immobilized on a surface, or by contact with a protein kinase C activator (*e.g.*, bryostatin) in conjunction with a calcium ionophore. For co-stimulation of an accessory molecule on the surface of the T cells, a ligand that binds the accessory molecule is used. For example, a population of $CD4^+$ cells can be contacted with an anti-CD3 antibody and an anti-CD28 antibody, under conditions appropriate for stimulating proliferation of the T cells. Similarly, to stimulate proliferation of $CD8^+$ T cells, an anti-CD3 antibody and the anti-CD28 antibody B-T3, XR-CD28 (Diaclone, Besançon, France) can be used as can other methods commonly known in the art (Berg *et al., Transplant Proc. 30*(8):3975-3977, 1998; Haanen *et al., J. Exp. Med. 190*(9):1319-1328, 1999; Garland *et al., J. Immunol Meth. 227*(1-2):53-63, 1999).

The primary stimulatory signal and the co-stimulatory signal for the T cell may be provided by different protocols. For example, the agents providing each signal may be in solution or coupled to a surface. When coupled to a surface, the agents may be coupled to the same surface (*i.e.*, in "cis" formation) or to separate surfaces (*i.e.*, in "trans" formation). Alternatively, one agent may be coupled to a surface and the other agent in solution. In one embodiment, the agent providing the co-stimulatory signal is bound to a cell surface and the agent providing the primary activation signal is in solution or coupled to a surface. In certain embodiments, both agents can be in solution. In another embodiment, the agents may be in soluble form, and then cross-linked to a surface, such as a cell expressing Fc receptors or an antibody or other binding agent which will bind to the agents. In one embodiment, the two agents are immobilized on beads, either on the same bead, *i.e.*, "cis," or to separate beads, *i.e.*, "trans." By way of example, the agent providing the primary activation signal is an anti-CD3 antibody and the agent providing the co-stimulatory signal is an anti-CD28 antibody; and both agents are co-immobilized to the same bead in equivalent molecular amounts. In one embodiment, a 1:1 ratio of each antibody bound to the beads for $CD4^+$ T cell expansion and T cell growth is used. In certain aspects of the present invention, a ratio of anti CD3:CD28 antibodies bound to the beads is used such

that an increase in T cell expansion is observed as compared to the expansion observed using a ratio of 1:1. In one particular embodiment an increase of from about .5 to about 3 fold is observed as compared to the expansion observed using a ratio of 1:1. In one embodiment, the ratio of CD3:CD28 antibody bound to the beads ranges from 100:1 to 1:100 and all integer values there between. In one aspect of the present invention, more anti-CD28 antibody is bound to the particles than anti-CD3 antibody, *i.e.* the ratio of CD3:CD28 is less than one. In certain embodiments of the invention, the ratio of anti CD28 antibody to anti CD3 antibody bound to the beads is greater than 2:1. In one embodiment, a 1:100 CD3:CD28 ratio of antibody bound to beads is used. In another embodiment, a 1:75 CD3:CD28 ratio of antibody bound to beads is used. In a further embodiment, a 1:50 CD3:CD28 ratio of antibody bound to beads is used. In another embodiment, a 1:30 CD3:CD28 ratio of antibody bound to beads is used. In one embodiment, a 1:10 CD3:CD28 ratio of antibody bound to beads is used. In another embodiment, a 1:3 CD3:CD28 ratio of antibody bound to the beads is used. In yet another embodiment, a 3:1 CD3:CD28 ratio of antibody bound to the beads is used.

Ratios of particles to cells from 1:500 to 500:1 and any integer values in between may be used to stimulate T cells or other target cells. As those of ordinary skill in the art can readily appreciate, the ratio of particle to cells may dependant on particle size relative to the target cell. For example, small sized beads could only bind a few cells, while larger beads could bind many. In certain embodiments the ratio of cells to particles ranges from 1:100 to 100:1 and any integer values in-between and in further embodiments the ratio comprises 1:9 to 9:1 and any integer values in between, can also be used to stimulate T cells. The ratio of anti-CD3- and anti-CD28-coupled particles to T cells that result in T cell stimulation can vary as noted above, however certain values include at least 1:20, 1:10, 1:9, 1:8, 1:7, 1:6, 1:5, 1:4, 1:3, 1:2, 1:1, 2:1, 3:1, 4:1, 5:1, 6:1, 7:1, 8:1, 9:1, 10:1, and 15:1 with one particular ratio being at least 1:1 particles per T cell. In one embodiment, a ratio of particles to cells of 1:1 or less is used. In one embodiment, the particle: cell ratio is 1:5. In further embodiments, the ratio of particles to cells can be varied depending on the day of stimulation. For example, in one embodiment, the ratio of particles to cells is from 1:1 to 10:1 on the first day and

additional particles are added to the cells every day or every other day thereafter for up to 10 days, at final ratios of from 1:1 to 1:10 (based on cell counts on the day of addition). In one embodiment, the ratio of particles to cells is 1:1 on the first day of stimulation and adjusted to 1:5 on the third and fifth days of stimulation. In another embodiment, particles are added on a daily or every other day basis to a final ratio of 1:1 on the first day, and 1:5 on the third and fifth days of stimulation. In another embodiment, the ratio of particles to cells is 2:1 on the first day of stimulation and adjusted to 1:10 on the third and fifth days of stimulation. In another embodiment, particles are added on a daily or every other day basis to a final ratio of 1:1 on the first day, and 1:10 on the third and fifth days of stimulation. One of skill in the art will appreciate that a variety of other ratios may be suitable for use in the present invention. In particular, ratios will vary depending on particle size and on cell size and type.

One aspect of the present invention stems from the surprising finding that using different bead:cell ratios can lead to different outcomes with respect to expansion of antigen-specific T cells. In particular, bead:cell ratios can be varied to selectively expand or delete antigen-specific (memory) T cells. In one embodiment, the particular bead:cell ratio used selectively deletes antigen-specific T cells. In a further embodiment, the particular bead:cell ratio used selectively expands antigen-specific T cells. The skilled artisan would readily appreciate that any ratio can be used as long as the desired expansion or deletion occurs. Therefore, the compositions and methods described herein can be used to expand specific populations of T cells, or to delete specific populations of T cells, for use in any variety of immunotherapeutic settings described herein

Using certain methodologies it may be advantageous to maintain long-term stimulation of a population of T cells following the initial activation and stimulation, by separating the T cells from the stimulus after a period of about 12 to about 14 days. The rate of T cell proliferation is monitored periodically (*e.g.*, daily) by, for example, examining the size or measuring the volume of the T cells, such as with a Coulter Counter. In this regard, a resting T cell has a mean diameter of about 6.8 microns, and upon initial activation and stimulation, in the presence of the stimulating

ligand, the T cell mean diameter will increase to over 12 microns by day 4 and begin to decrease by about day 6. When the mean T cell diameter decreases to approximately 8 microns, the T cells may be reactivated and re-stimulated to induce further proliferation of the T cells. Alternatively, the rate of T cell proliferation and time for T cell re-stimulation can be monitored by assaying for the presence of cell surface molecules, such as, CD154, CD54, CD25, CD137, CD134, , which are induced on activated T cells.

In one embodiment, T cell stimulation is performed with anti-CD3 and anti-CD28 antibodies co-immobilized on beads (3x28 beads), for a period of time sufficient for the cells to return to a quiescent state (low or no proliferation) (approximately 8-14 days after initial stimulation). The stimulation signal is then removed from the cells and the cells are washed and infused back into the patient. The cells at the end of the stimulation phase are rendered "super-inducible" by the methods of the present invention, as demonstrated by their ability to respond to antigens and the ability of these cells to demonstrate a memory-like phenotype, as is evidence by the examples. Accordingly, upon re-stimulation either exogenously or by an antigen *in vivo* after infusion, the activated T cells demonstrate a robust response characterized by unique phenotypic properties, such as sustained CD154 expression and increased cytokine production.

In further embodiments of the present invention, the cells, such as T cells, are combined with agent-coated beads, the beads and the cells are subsequently separated, and then the cells are cultured. In an alternative embodiment, prior to culture, the agent-coated beads and cells are not separated but are cultured together. In a further embodiment, the beads and cells are first concentrated by application of a force, resulting in cell surface moiety ligation, thereby inducing cell stimulation.

By way of example, when T cells are the target cell population, the cell surface moieties may be ligated by allowing paramagnetic beads to which anti-CD3 and anti-CD28 are attached (3x28 beads) to contact the T cells. In one embodiment the cells (for example, 10^4 to 10^9 T cells) and beads (for example, DYNABEADS® M-450 CD3/CD28 T paramagnetic beads at a ratio of 1:1) are combined in a buffer, preferably

PBS (without divalent cations such as, calcium and magnesium). Again, those of ordinary skill in the art can readily appreciate any cell concentration may be used. For example, the target cell may be very rare in the sample and comprise only 0.01% of the sample or the entire sample (*i.e.* 100%) may comprise the target cell of interest. Accordingly, any cell number is within the context of the present invention. In certain embodiments, it may be desirable to significantly decrease the volume in which particles and cells are mixed together (*i.e.*, increase the concentration of cells), to ensure maximum contact of cells and particles. For example, in one embodiment, a concentration of about 2 billion cells/ml is used. In another embodiment, greater than 100 million cells/ml is used. In a further embodiment, a concentration of cells of 10, 15, 20, 25, 30, 35, 40, 45, or 50 million cells/ml is used. In yet another embodiment, a concentration of cells from 75, 80, 85, 90, 95, or 100 million cells/ml is used. In further embodiments, concentrations of 125 or 150 million cells/ml can be used. Using high concentrations can result in increased cell yield, cell activation, and cell expansion. Further, use of high cell concentrations allows more efficient capture of cells that may weakly express target antigens of interest, such as CD28-negative T cells. Such populations of cells may have therapeutic value and would be desirable to obtain. For example, using high concentration of cells allows more efficient selection of CD8+ T cells that normally have weaker CD28 expression.

In a related embodiment, it may be desirable to use lower concentrations of cells. By significantly diluting the mixture of T cells and particles, interactions between particles and cells is minimized. This selects for cells that express high amounts of desired antigens to be bound to the particles. For example, CD4+ T cells express higher levels of CD28 and are more efficiently captured and stimulated than CD8+ T cells in dilute concentrations. In one embodiment, the concentration of cells used is about 5 X 10^6/ml. In other embodiments, the concentration used can be from about 1 X 10^5/ml to about 1 X 10^6/ml, and any integer value in between.

The buffer that the cells are suspended in may be any that is appropriate for the particular cell type. When utilizing certain cell types the buffer may contain other components, *e.g.* 1-5% serum, necessary to maintain cell integrity during the

process. In another embodiment, the cells and beads may be combined in cell culture media. The cells and beads may be mixed, for example, by rotation, agitation or any means for mixing, for a period of time ranging from one minute to several hours. The container of beads and cells is then concentrated by a force, such as placing in a magnetic field. Media and unbound cells are removed and the cells attached to the beads are washed, for example, by pumping via a peristaltic pump, and then resuspended in media appropriate for cell culture.

In one embodiment of the present invention, the mixture may be cultured for several hours (about 3 hours) to about 14 days or any hourly integer value in between. In another embodiment, the mixture may be cultured for 21 days. In one embodiment of the invention the beads and the T cells are cultured together for about eight days. In another embodiment, the beads and T cells are cultured together for 2-3 days. Several cycles of stimulation may also be desired such that culture time of T cells can be 60 days or more. Conditions appropriate for T cell culture include an appropriate media (*e.g.*, Minimal Essential Media or RPMI Media 1640 or, X-vivo 15, (BioWhittaker)) that may contain factors necessary for proliferation and viability, including serum (*e.g.*, fetal bovine or human serum), interleukin-2 (IL-2), insulin, IFN-γ, IL-4, GM-CSF, IL-10, IL-12, TGFβ, and TNF-α. or any other additives for the growth of cells known to the skilled artisan. Other additives for the growth of cells include, but are not limited to, surfactant, plasmanate, and reducing agents such as N-acetyl-cysteine and 2-mercaptoethanol. Media can include RPMI 1640, AIM-V, DMEM, MEM, α-MEM, F-12, X-Vivo 15, and X-Vivo 20, with added amino acids and vitamins, either serum-free or supplemented with an appropriate amount of serum (or plasma) or a defined set of hormones, and/or an amount of cytokine(s) sufficient for the growth and expansion of T cells. Antibiotics, *e.g.*, penicillin and streptomycin, are included only in experimental cultures, not in cultures of cells that are to be infused into a subject. The target cells are maintained under conditions necessary to support growth, for example, an appropriate temperature (*e.g.*, 37° C) and atmosphere (*e.g.*, air plus 5% $C0_2$).

When using a magnetic field as the concentrating force the magnetic field strength applied to the cells prior to cell culture may be between the range of 200 gauss to 12,000 gauss on the magnetic surface. The shape and size of the magnet may be adapted to the size and shape of the mixing or cell culture vessels or to any other parameter that facilitates or increases cell to cell contact and concentration of the cells. The magnetic force may be diffused by placing a material that acts as a buffer or spacer between the magnet and the paramagnetic beads contained within the mixture with cells. A strong magnetic force is generally considered to be at least 7500 gauss at the surface, whereas a weak magnetic force is considered to be in the range of 2000-2500 gauss at the surface. The approximate magnetic force applied by a magnet on a paramagnetic bead depends upon the volume of the paramagnetic bead and the magnetic field strength according to the following formula:

$$F_{mag} = (v)\ (\psi)\ (B)\ (dB/dx)$$

where F_{mag} equals the magnetic force, v equals the volume of the paramagnetic bead, ψ equals the magnetic susceptibility of a paramagnetic bead (a value provided by the manufacturer), B equals the magnetic field strength, and (dB/dx) equals the field strength gradient. One of skill in the art will appreciate that the factors on the right-hand side of the equation can be obtained or measured, allowing the magnetic force applied to be calculated.

Cells stimulated by the methods of the present invention are activated as shown by the induction of signal transduction, expression of cell surface markers and/or proliferation. One such marker appropriate for T cells is CD154 which is an important immunomodulating molecule. The expression of CD154 is extremely beneficial in amplifying the immune response. CD154 interacts with the CD40 molecule expressed on many B cells, dendritic cells, monocytes, and some endothelial cells. Accordingly, this unexpected and surprising increase in CD154 expression is likely to lead to more efficacious T cell compositions. Stimulation of $CD3^+$ cells as described herein provides T cells that express a 1.1 to 20-fold increases in the levels of certain cell surface markers such as CD154 expression on days 1, 2, 3, or 4 following stimulation. (See

Example 5, Table 2 and Figure 4.) Expression of another cell surface marker, CD25, also was greater on T cells after concentration and stimulation than on cells prior to culture or cells stimulated by other methods. (See Table 2.)

One of skill in the art will appreciate that any target cell that can be stimulated by cell surface moiety ligation may be combined with the agent-coated surface, such as beads. Further, the agent-coated surfaces, such as, beads may be separated from the cells prior to culture, at any point during culture, or at the termination of culture. In addition, the agent-coated surfaces ligated to the target cells may be separated from the non-binding cells prior to culture or the other cells may remain in culture as well. In one embodiment, prior to culture, the agent-coated beads and target cells are not separated but are cultured together. In a further embodiment, the beads and target cells are first concentrated by application of a force, resulting in cell surface moiety ligation, thereby inducing stimulation and subsequent activation.

Also contemplated by this invention, are other means to increase the concentration of the target cells, for example, a T cell fraction bound to a surface coated with primary and secondary stimulatory molecules. In addition to application of a magnetic force, other forces greater than gravitational force may be applied, for example, but not limited to, centrifugal force, transmembrane pressure, and a hydraulic force. Concentration may also be accomplished by filtration.

One of skill in the art will readily appreciate that contact between the agent-coated beads and the cells to be stimulated can be increased by concentration using other forces. Accordingly, any means for concentrating cells with cell surface moiety binding ligands will be sufficient as long as the concentration brings together cells and agents in a manner that exceeds gravity or diffusion.

It should be understood that in various embodiments the agent-coated surface may be a particle, such as a bead which is mixed with the cells and concentrated in a small volume in a magnetic field, thus drawing all the particles and particle bound cells into a defined and concentrated area. In certain embodiments, the agent-coated surface may be drawn together by force within thirty seconds to four hours of being exposed to the target cells. In other embodiments the time may be from 1 minute to 2

hours, or all integer ranges in between. Application of a force to a cell population with receptor bearing cells that is mixed with a surface to which at least one cell surface ligand is attached may induce cell receptor polarization, aggregating cell surface molecules. This means for inducing cell surface polarization may enhance signaling within the cell by aggregating cell surface molecules that comprise lipid rafts. Such aggregation can induce a signal pathway, which may lead to down-regulation or suppression of a cellular event. Alternatively, the aggregation of cell surface molecules may lead to up-regulation or activation of a cellular event.

A cellular event may include, for example, receptor-mediated signal transduction that induces or suppresses a particular pathway, including an apoptotic pathway, or induces phosphorylation of proteins, or stimulates or suppresses growth signals. In one embodiment, the cells may be lymphocytes, particularly a T cell, and the cell surface ligand may be an anti-CD3 antibody attached to a surface, for example, a particle. The particle may be a paramagnetic bead and the force applied a magnetic force. Application of a magnetic force to a mixture of the lymphocytes and anti-CD3-coated surface of the paramagnetic bead may cause the CD3 receptors of the T cell to polarize more quickly than would occur in the absence of an external force. This method of stimulating the T cell promotes more rapid activation of the T cell immune response pathways and proliferation of cells.

In one embodiment of the present invention, bead:cell ratios can be tailored to obtain a desired T cell phenotype. In one embodiment, bead:cell ratios can be vaired to selectively expand or delete antigen-specific (memory) T cells. In one embodiment, the particular bead:cell ratio used selectively deletes antigen-specific T cells. In a further embodiment, the particular bead:cell ratio used selectively expands antigen-specific T cells. The skilled artisan would readily appreciate that any ratio can be used as long as the desired expansion or deletion of antigen-specific T cells occurs. Therefore, the compositions and methods described herein can be used to expand specific populations of T cells, or to delete specific populations of T cells, for use in any variety of immunotherapeutic settings described herein.

In another embodiment, the time of exposure to stimulatory agents such as anti-CD3/anti-CD28 (*i.e.*, 3x28)-coated beads may be modified or tailored to obtain a desired T cell phenotype. Alternatively, a desired population of T cells can be selected using any number of selection techniques, prior to stimulation. One may desire a greater population of helper T cells (T_H), typically $CD4^+$ as opposed to $CD8^+$ cytotoxic or regulatory T cells, because an expansion of T_H cells could improve or restore overall immune responsiveness. While many specific immune responses are mediated by $CD8^+$ antigen-specific T cells, which can directly lyse or kill target cells, most immune responses require the help of $CD4^+$ T cells, which express important immune-regulatory molecules, such as GM-CSF, CD40L, and IL-2, for example. Where CD4-mediated help is preferred, a method, such as that described herein, which preserves or enhances the CD4:CD8 ratio could be of significant benefit. Increased numbers of $CD4^+$ T cells can increase the amount of cell-expressed CD40L introduced into patients, potentially improving target cell visibility (improved APC function). Similar effects can be seen by increasing the number of infused cells expressing GM-CSF, or IL-2, all of which are expressed predominantly by $CD4^+$ T cells. Alternatively, in situations where CD4-help is needed less and increased numbers of $CD8^+$ T cells are desirous, the XCELLERATE approaches described herein can also be utilized, by for example, pre-selecting for $CD8^+$ cells prior to stimulation and/or culture. Such situations may exist where increased levels of IFN-γ or increased cytolysis of a target cell is preferred.

To effectuate isolation of different T cell populations, exposure times to the to the particles may be varied. For example, in one embodiment, T cells are isolated by incubation with 3x28 beads, such as Dynabeads M-450, for a time period sufficient for positive selection of the desired T cells. In one embodiment, the time period is about 30 minutes. In a further embodiment, the time period is at least 1, 2, 3, 4, 5, or 6 hours. In yet another embodiment, the time period is 10 to 24 hours or more. In one embodiment, the incubation time period is 24 hours. For isolation of T cells from cancer patients, use of longer incubation times, such as 24 hours, can increase cell yield.

To effectuate isolation of different T cell populations, exposure times to the concentration force may be varied or pulsed. For example when such force is a

magnet, exposure to the magnet or the magnetic field strength may be varied, and/or expansion times may be varied to obtain the specific phenotype of interest. The expression of a variety of phenotypic markers change over time; therefore, a particular time point may be chosen to obtain a specific population of T cells. Accordingly, depending on the cell type to be stimulated, the stimulation and/or expansion time may be 10 weeks or less, 8 weeks or less, four weeks or less, 2 weeks or less, 10 days or less, or 8 days or less (four weeks or less includes all time ranges from 4 weeks down to 1 day (24 hours) or any value between these numbers). In some embodiments in may be desirable to clone T cells using, for example, limiting dilution or cell sorting, wherein longer stimulation time may be necessary. In some embodiments, stimulation and expansion may be carried out for 6 days or less, 4 days or less, 2 days or less, and in other embodiments for as little as 24 or less hours, and preferably 4-6 hours or less (these ranges include any integer values in between). When stimulation of T cells is carried out for shorter periods of time, the population of T cells may not increase in number as dramatically, but the population will provide more robust and healthy activated T cells that can continue to proliferate *in vivo* and more closely resemble the natural effector T cell pool. As the availability of T cell help is often the limiting factor in antibody responses to protein antigens, the ability to selectively expand or selectively infuse a $CD4^+$ rich population of T cells into a subject is extremely beneficial. Further benefits of such enriched populations are readily apparent in that activated helper T cells that recognize antigens presented by B lymphocytes deliver two types of stimuli, physical contact and cytokine production, that result in the proliferation and differentiation of B cells.

T cells that have been exposed to varied stimulation times may exhibit different characteristics. For example, typical blood or apheresed peripheral blood mononuclear cell products have a helper T cell population(T_H, $CD4^+$) that is greater than the cytotoxic or suppressor T cell population (T_C, $CD8^+$). *Ex vivo* expansion of T cells by stimulating CD3 and CD28 receptors produces a population of T cells that prior to about days 8-9 consists predominately of T_H cells, while after about days 8-9, the population of T cells comprises an increasingly greater population of T_C cells.

Accordingly, depending on the purpose of treatment, infusing a subject with a T cell population comprising predominately of T_H cells may be advantageous. Similarly, if an antigen-specific subset of T_C cells has been isolated it may be beneficial to expand this subset to a greater degree.

Further, in addition to CD4 and CD8 markers, other phenotypic markers vary significantly, but in large part, reproducibly during the course of the cell expansion process. Thus, such reproducibility enables the ability to tailor an activated T cell product for specific purposes.

In one such example, among the important phenotypic markers that reproducibly vary with time are the high affinity IL-2 receptor (CD25), CD40 ligand (CD154), and CD45RO (a molecule that by preferential association with the TCR may increase the sensitivity of the TCR to antigen binding). As one of ordinary skill in the art readily appreciates, such molecules are important for a variety of reasons. For example, CD25 constitutes an important part of the autocrine loop that allows rapid T cell division. CD154 has been shown to play a key role in stimulating maturation of the antigen-presenting dendritic cells; activating B-cells for antibody production; regulating T_H cell proliferation; enhancing T_C cell differentiation; regulating cytokine secretion of both T_H cells and antigen-presenting cells; and stimulating expression of co-stimulatory ligands, including CD80, CD86, and CD154.

Cytokine production peaks in the first few days of the *ex vivo* expansion process. Accordingly, because cytokines are known to be important for mediating T cell activation and function as well as immune response modulation, such cytokines are likely critical in the development of a therapeutic T cell product, that is able to undergo reactivation upon contact with an additional antigen challenge. Cytokines important in this regard, include, but are not limited to, IL-2, IL-4, TNF-α, and IFN-γ. Thus, by obtaining a population of T cells during the first few days of expansion and infusing these cells into a subject, a therapeutic benefit may occur in which additional activation and expansion of T cells *in vivo* occurs.

In addition to the cytokines and the markers discussed previously, expression of adhesion molecules known to be important for mediation of T cell

activation and immune response modulation also change dramatically but reproducibly over the course of the *ex vivo* expansion process. For example, CD62L is important for homing of T cells to lymphoid tissues and trafficking T cells to sites of inflammation. Under certain circumstances of disease and injury, the presence of activated T cells at these sites may be disadvantageous. Because down-regulation of CD62L occurs early following activation, the T cells could be expanded for shorter periods of time. Conversely, longer periods of time in culture would generate a T cell population with higher levels of CD62L and thus a higher ability to target the activated T cells to these sites under other conditions. Another example of a polypeptide whose expression varies over time is CD49d, an adhesion molecule that is involved in trafficking lymphocytes from blood to tissues spaces at sites of inflammation. Binding of the CD49d ligand to CD49d also allows the T cell to receive co-stimulatory signals for activation and proliferation through binding by VCAM-1 or fibronectin ligands. The expression of the adhesion molecule CD54, involved in T cell-APC and T cell-T cell interactions as well as homing to sites of inflammation, also changes over the course of expansion. Accordingly, T cells could be stimulated for selected periods of time that coincide with the marker profile of interest and subsequently collected and infused. Thus, T cell populations could be tailored to express the markers believed to provide the most therapeutic benefit for the indication to be treated.

In the various embodiments, one of ordinary skill in the art understands removal of the stimulation signal from the cells is dependent upon the type of surface used. For example, if paramagnetic beads are used, then magnetic separation is the feasible option. Separation techniques are described in detail by paramagnetic bead manufacturers' instructions (for example, DYNAL Inc., Oslo, Norway). Furthermore, filtration may be used if the surface is a bead large enough to be separated from the cells. In addition, a variety of transfusion filters are commercially available, including 20 micron and 80 micron transfusion filters (Baxter). Accordingly, so long as the beads are larger than the mesh size of the filter, such filtration is highly efficient. In a related embodiment, the beads may pass through the filter, but cells may remain, thus allowing

separation. In one embodiment the biocompatible surface used degrades (*i.e.* biodegradable) in culture during the exposure period.

Those of ordinary skill in the art will readily appreciate that the cell stimulation methodologies described herein may be carried out in a variety of environments (*i.e.*, containers). For example, such containers may be culture flasks, culture bags, or any container capable of holding cells, preferably in a sterile environment. In one embodiment of the present invention a bioreactor is also useful. For example, several manufacturers currently make devices that can be used to grow cells and be used in combination with the methods of the present invention. See for example, Celdyne Corp., Houston, TX; Unisyn Technologies, Hopkinton, MA; Synthecon, Inc. Houston, TX; Aastrom Biosciences, Inc. Ann Arbor, MI; Wave Biotech LLC, Bedminster, NJ. Further, patents covering such bioreactors include U.S. Patent Nos: 6,096,532; 5,985,653; 5,888,807; 5,190,878, which are incorporated herein by reference.

In one embodiment, the magnet used for simultaneous stimulation and concentration of the cells of the present invention may be incorporated into the base rocker platform of a bioreactor device, such as "The Wave" (Wave Biotech LLC, Bedminster, NJ). The magnet, or a magnetizable element, may also be enclosed into a standard bioreactor vessel such as a cylindrical application unit. This built-in magnetic element may be capable of being switched on and off as desired at various points in the cell culture procedure. The integrated magnet, or magnetizable element, is positioned so as to allow a magnetic field emanating therefrom to pass through the culture vessel. In certain embodiments, the magnet, or magnetizable element, is incorporated within a wall, or alternatively, within the body of the culture vessel. In a further embodiment, the cells can be magnetically concentrated and/or activated, magnetically separated or isolated at a desired point during culture without the need to transfer cells to a different culture or magnetic separation unit. Use of such a built-in magnetic element can facilitate culture, stimulation and concentration, and separation processes to enable expansion and tailoring of specific functional cell populations for immunotherapeutic infusion into patients in cell or gene-based therapies. Further, this device provides an

improved means for specific production of molecules both inside cells and their secretion to the outside of cells.

The integrated magnetic or magnetizable device as described above can be used to either remove magnetic particles from the culture, retaining them in the culture vessel, whilst the desired cells and/or desired molecules present in the culture media are removed. Alternatively, the cells and/or desired molecules may be specifically retained in the culture bag, or other suitable culture vessel, by interaction with magnetic particles that have been coated with specific molecules as described herein that bind to the desired cells and/or molecules. The built-in magnetic or magnetizable device enables the washing of cell populations and replacement of media in the cell culture bag by magnetically immobilizing/concentrating cells with specific particles and flowing media and or other solutions through the bag. This device effectively eliminates the need for a separate magnetic separation device by providing a fully integrated system, thereby reducing process time and manual operations for tubing connectors, reducing the number of containers used in processing and reducing the likelihood of contamination through the number of tube and container connections required. This integrated magnetic or magnetizable device-culture system also reduces the volumes needed in the culture processing and formulation.

As mentioned previously, one aspect of the present invention is directed to the surprising finding that the combination of a force which induces the concentration of cells, ligation of cell surface moieties, and culturing cells in a rocking, closed system, results in a profound enhancement in activation and expansion of these cells. Accordingly, in one embodiment, a bioreactor with a base rocker platform is used, for example such as "The Wave" (Wave Biotech LLC, Bedminster, NJ), that allows for varying rates of rocking and at a variety of different rocking angles. The skilled artisan will recognize that any platform that allows for the appropriate motion for optimal expansion of the cells is within the context of the present invention. In certain embodiments, the methods of stimulation and expansion of the present invention provide for rocking the culture container during the process of culturing at 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, or 20 rocks per minute.

In certain embodiments, the capacity of the bioreactor container ranges from about 0.1 liter to about 200 liters of medium. The skilled artisan will readily appreciate that the volume used for culture will vary depending on the number of starting cells and on the final number of cells desired. In particular embodiments, the cells of the present invention, such as T cells are seeded at an initial concentration of about 0.2 X 10^6 cells/ml to about 5 X 10^6 cells/ml, and any concentration therebetween. In one embodiment, the cells may be cultured initially in a static environment and transferred to a bioreactor on a rocking platform after 1, 2, 3, 4, 5, 6, 7, 8, or more days of culture. In a related embodiment, the entire process of stimulation, activation, and expansion takes place in a bioreactor comprising a rocking platform and an integrated magnet, as described above. Illustrative bioreactors include, but are not limited to, "The Wave".

In one embodiment, the cell stimulation methods of the present invention are carried out in a closed system, such as a bioreactor, that allows for perfusion of medium at varying rates, such as from about 0.1 ml/minute to about 3 ml/minute. Accordingly, in certain embodiments, the container of such a closed system comprises an outlet filter, an inlet filter, and a sampling port for sterile transfer to and from the closed system. In other embodiments, the container of such a closed system comprises a syringe pump and control for sterile transfer to and from the closed system. Further embodiments provide for a mechanism, such as a load cell, for controlling media in-put and out-put by continuous monitoring of the weight of the bioreactor container. In one embodiment the system comprises a gas manifold. In another embodiment, the bioreactor of the present invention comprises a CO_2 gas mix rack that supplies a mixture of ambient air and CO_2 to the bioreactor container and maintains the container at positive pressure. In another embodiment, the bioreactor of the present invention comprises a variable heating element.

In one embodiment, media is allowed to enter the container starting on day 2, 3, 4, 5, or 6 at about 0.5 to 5.0 liters per day until the desired final volume is achieved. In one embodiment, media enters the container at 2 liters per day starting at day 4, until the volume reaches 10 liters. Once desired volume is achieved, perfusion of

media can be initiated. In certain embodiments, perfusion of media through the system is initiated on about day 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, or 12 of culture. In one embodiment, perfusion is initiated when the volume is at about 0.1 liter to about 200 liters of media. In one embodiment, perfusion is initiated when the final volume is at 4, 5, 6, 7, 8, 9, 10, or 20 liters.

In a further embodiment of the present invention, the cells, such as T cells, are cultured for up to 5 days in a closed, static system and then transferred to a closed system that comprises a rocking element to allow rocking of the culture container at varying speeds.

In certain aspects, the methodologies of the present invention provide for the expansion of cells, such as T cells, to a concentration of about between 6 X 10^6 cell/ml and about 90 X 10^6 cells/ml in less that about two weeks. In particular the methodologies herein provide for the expansion of T cells to a concentration of about 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, or 85 X 10^6 cells/ml and all concentrations therein. In certain embodiments, the cells reach a desired concentration, such as any of those listed above, by about day 5, 6, 7, 8, 9, 10, 11, or 12 of culture. In one embodiment, the T cells expand by at least about 1.5 fold in about 24 hours from about day 4 to about day 12 of culture. In one embodiment, the cells, such as T cells, expand from a starting number of cells of about 100 X 10^6 to a total of about 500 X 10^9 cells in less than about two weeks. In further embodiments, the T cells expand from a starting number of cells of about 500 X 10^6 to a total of about 500 X 10^9 cells in less than about two weeks. In related embodiments, the cells expand from a starting number of about 100 – 500 X 10^6 to a total of about 200, 300, or 400 X 10^9 cells in less than about two weeks.

In further embodiments of the present invention, the cell activation and expansion methods described herein and the conditioned medium generated using these methods can be used for the production of exosomes. In cells, vesicles can be formed by budding of the endosomal membrane into the lumen of the compartment; this process results in the formation of multivesicular bodies (MVBs). Fusion of MVBs with the plasma membrane results in secretion of the small internal vesicles, termed

exosomes. The conditioned medium can be used for the culture of other T cells or for the culture of other types cells.

Although the antibodies used in the methods described herein can be readily obtained from public sources, such as the ATCC, antibodies to T cell accessory molecules and the CD3 complex can be produced by standard techniques. Methodologies for generating antibodies for use in the methods of the invention are well-known in the art and are discussed in further detail herein.

Ligand Immobilization on a Surface

As indicated above, the methods of the present invention preferably use ligands bound to a surface. The surface may be any surface capable of having a ligand bound thereto or integrated into and that is biocompatible, that is, substantially non-toxic to the target cells to be stimulated. The biocompatible surface may be biodegradable or non-biodegradable. The surface may be natural or synthetic, and a synthetic surface may be a polymer. The surface may comprise collagen, purified proteins, purified peptides, polysaccharides, glycosaminoglycans, or extracellular matrix compositions. A polysaccharide may include for example, cellulose, agarose, dextran, chitosan, hyaluronic acid, or alginate. Other polymers may include polyesters, polyethers, polyanhydrides, polyalkylcyanoacryllates, polyacrylamides, polyorthoesters, polyphosphazenes, polyvinylacetates, block copolymers, polypropylene, polytetrafluorethylene (PTFE), or polyurethanes. The polymer may be lactic acid or a copolymer. A copolymer may comprise lactic acid and glycolic acid (PLGA). Non-biodegradable surfaces may include polymers, such as poly(dimethylsiloxane) and poly(ethylene-vinyl acetate). Biocompatible surfaces include for example, glass (*e.g.*, bioglass), collagen, metal, hydroxyapatite, aluminate, bioceramic materials, hyaluronic acid polymers, alginate, acrylic ester polymers, lactic acid polymer, glycolic acid polymer, lactic acid/glycolic acid polymer, purified proteins, purified peptides, or extracellular matrix compositions. Other polymers comprising a surface may include glass, silica, silicon, hydroxyapatite, hydrogels, collagen, acrolein, polyacrylamide, polypropylene, polystyrene, nylon, or any number

of plastics or synthetic organic polymers, or the like. The surface may comprise a biological structure, such as a liposome or a cell. The surface may be in the form of a lipid, a plate, bag, pellet, fiber, mesh, or particle. A particle may include, a colloidal particle, a microsphere, nanoparticle, a bead, or the like. In the various embodiments, commercially available surfaces, such as beads or other particles, are useful (*e.g.*, Miltenyi Particles, Miltenyi Biotec, Germany; Sepharose beads, Pharmacia Fine Chemicals, Sweden; DYNABEADS™, Dynal Inc., New York; PURABEADS™, Prometic Biosciences).

When beads are used, the bead may be of any size that effectuates target cell stimulation. In one embodiment, beads are preferably from about 5 nanometers to about 500 μm in size. Accordingly, the choice of bead size depends on the particular use the bead will serve. For example, if the bead is used for monocyte depletion, a small size is chosen to facilitate monocyte ingestion (*e.g.*, 2.8 μm and 4.5 μm in diameter or any size that may be engulfed, such as nanometer sizes); however, when separation of beads by filtration is desired, bead sizes of no less than 50 μm are typically used. Further, when using paramagnetic beads, the beads typically range in size from about 2.8 μm to about 500 μm and more preferably from about 2.8 μm to about 50 μm. Lastly, one may choose to use super-paramagnetic nanoparticles which can be as small as about 10^{-5} nm. Accordingly, as is readily apparent from the discussion above, virtually any particle size may be utilized.

An agent may be attached or coupled to, or integrated into a surface by a variety of methods known and available in the art. The agent may be a natural ligand, a protein ligand, or a synthetic ligand. The attachment may be covalent or noncovalent, electrostatic, or hydrophobic and may be accomplished by a variety of attachment means, including for example, chemical, mechanical, enzymatic, electrostatic, or other means whereby a ligand is capable of stimulating the cells. For example, the antibody to a ligand first may be attached to a surface, or avidin or streptavidin may be attached to the surface for binding to a biotinylated ligand. The antibody to the ligand may be attached to the surface via an anti-idiotype antibody. Another example includes using protein A or protein G, or other non-specific antibody binding molecules, attached to

surfaces to bind an antibody. Alternatively, the ligand may be attached to the surface by chemical means, such as cross-linking to the surface, using commercially available cross-linking reagents (Pierce, Rockford, IL) or other means. In certain embodiments, the ligands are covalently bound to the surface. Further, in one embodiment, commercially available tosyl-activated DYNABEADS™ or DYNABEADS™ with epoxy-surface reactive groups are incubated with the polypeptide ligand of interest according to the manufacturer's instructions. Briefly, such conditions typically involve incubation in a phosphate buffer from pH 4 to pH 9.5 at temperatures ranging from 4 to 37 degrees C.

In one aspect, the agent, such as certain ligands may be of singular origin or multiple origins and may be antibodies or fragments thereof while in another aspect, when utilizing T cells, the co-stimulatory ligand is a B7 molecule (*e.g.*, B7-1, B7-2). These ligands are coupled to the surface by any of the different attachment means discussed above. The B7 molecule to be coupled to the surface may be isolated from a cell expressing the co-stimulatory molecule, or obtained using standard recombinant DNA technology and expression systems that allow for production and isolation of the co-stimulatory molecule(s) as described herein. Fragments, mutants, or variants of a B7 molecule that retain the capability to trigger a co-stimulatory signal in T cells when coupled to the surface of a cell can also be used. Furthermore, one of ordinary skill in the art will recognize that any ligand useful in the activation and induction of proliferation of a subset of T cells may also be immobilized on beads or culture vessel surfaces or any surface. In addition, while covalent binding of the ligand to the surface is one methodology, adsorption or capture by a secondary monoclonal antibody may also be used. The amount of a particular ligand attached to a surface may be readily determined by flow cytometric analysis if the surface is that of beads or determined by enzyme-linked immunosorbent assay (ELISA) if the surface is a tissue culture dish, mesh, fibers, bags, for example.

In a particular embodiment, the stimulatory form of a B7 molecule or an anti-CD28 antibody or fragment thereof is attached to the same solid phase surface as the agent that stimulates the TCR/CD3 complex, such as an anti-CD3 antibody. In an

additional embodiment, the stimulatory form of a 4-1BB molecule or an anti-4-1BB antibody or fragment thereof is attached to the same solid phase surface as the agent that stimulates the TCR/CD3 complex, such as an anti-CD3 antibody. In addition to anti-CD3 antibodies, other antibodies that bind to receptors that mimic antigen signals may be used. For example, the beads or other surfaces may be coated with combinations of anti-CD2 antibodies and a B7 molecule and in particular anti-CD3 antibodies and anti-CD28 antibodies. In further embodiments, the surfaces may be coated with three or more agents, such as combinations of any of the agents described herein, for example, anti-CD3 antibodies, anti-CD28 antibodies, and anti-4-1BB antibodies.

When coupled to a surface, the agents may be coupled to the same surface (*i.e.*, in "cis" formation) or to separate surfaces (*i.e.*, in "trans" formation). Alternatively, one agent may be coupled to a surface and the other agent in solution. In one embodiment, the agent providing the co-stimulatory signal is bound to a cell surface and the agent providing the primary activation signal is in solution or coupled to a surface. In one embodiment, the two agents are immobilized on beads, either on the same bead, *i.e.*, "cis," or to separate beads, *i.e.*, "trans." By way of example, the agent providing the primary activation signal is an anti-CD3 antibody and the agent providing the co-stimulatory signal is an anti-CD28 antibody; and both agents are co-immobilized to the same bead in equivalent molecular amounts. In one embodiment, a 1:1 ratio of each antibody bound to the beads for $CD4^+$ T cell expansion and T cell growth is used. In certain aspects of the present invention, a ratio of anti CD3:CD28 antibodies bound to the beads is used such that an increase in T cell expansion is observed as compared to the expansion observed using a ratio of 1:1. In one embodiment an increase of from about .5 to about 3 fold is observed as compared to the expansion observed using a ratio of 1:1. In one embodiment, the ratio of CD3:CD28 antibody bound to the beads ranges from 100:1 to 1:100 and all integer values there between. In one aspect of the present invention, more anti-CD28 antibody is bound to the particles than anti-CD3 antibody, *i.e.* the ratio of CD3:CD28 is less than one. In certain embodiments of the invention, the ratio of anti CD28 antibody to anti CD3 antibody bound to the beads is greater than

2:1. In one embodiment, a 1:100 CD3:CD28 ratio of antibody bound to beads is used. In another embodiment, a 1:75 CD3:CD28 ratio of antibody bound to beads is used. In a further embodiment, a 1:50 CD3:CD28 ratio of antibody bound to beads is used. In another embodiment, a 1:30 CD3:CD28 ratio of antibody bound to beads is used. In one embodiment, a 1:10 CD3:CD28 ratio of antibody bound to beads is used. In another embodiment, a 1:3 CD3:CD28 ratio of antibody bound to the beads is used. In yet another embodiment, a 3:1 CD3:CD28 ratio of antibody bound to the beads is used.

In certain aspects of the present invention, three or more agents are coupled to a surface. In certain embodiments, the agents may be coupled to the same surface (*i.e.*, in "cis" formation) or to separate surfaces (*i.e.*, in "trans" formation). Alternatively, one or more agents may be coupled to a surface and the other agent or agents may be in solution.

Agents

Agents contemplated by the present invention include protein ligands, natural ligands, and synthetic ligands. Agents that can bind to cell surface moieties, and under certain conditions, cause ligation and aggregation that leads to signaling include, but are not limited to, lectins (for example, PHA, lentil lectins, concanavalin A), antibodies, antibody fragments, peptides, polypeptides, glycopeptides, receptors, B cell receptor and T cell receptor ligands, extracellular matrix components, steroids, hormones (for example, growth hormone, corticosteroids, prostaglandins, tetra-iodo thyronine), bacterial moieties (such as lipopolysaccharides), mitogens, antigens, superantigens and their derivatives, growth factors, cytokine, viral proteins (for example, HIV gp-120), adhesion molecules (such as, L-selectin, LFA-3, CD54, LFA-1), chemokines, and small molecules. The agents may be isolated from natural sources such as cells, blood products, and tissues, or isolated from cells propagated *in vitro*, or prepared recombinantly, or by other methods known to those with skill in the art.

In one aspect of the present invention, when it is desirous to stimulate T cells, useful agents include ligands that are capable of binding the CD3/TCR complex, CD2, and/or CD28 and initiating activation or proliferation, respectively. Accordingly,

the term ligand includes those proteins that are the "natural" ligand for the cell surface protein, such as a B7 molecule for CD28, as well as artificial ligands such as antibodies directed to the cell surface protein. Such antibodies and fragments thereof may be produced in accordance with conventional techniques, such as hybridoma methods and recombinant DNA and protein expression techniques. Useful antibodies and fragments may be derived from any species, including humans, or may be formed as chimeric proteins, which employ sequences from more than one species.

Methods well known in the art may be used to generate antibodies, polyclonal antisera, or monoclonal antibodies that are specific for a ligand. Antibodies also may be produced as genetically engineered immunoglobulins (Ig) or Ig fragments designed to have desirable properties. For example, by way of illustration and not limitation, antibodies may include a recombinant IgG that is a chimeric fusion protein having at least one variable (V) region domain from a first mammalian species and at least one constant region domain from a second distinct mammalian species. Most commonly, a chimeric antibody has murine variable region sequences and human constant region sequences. Such a murine/human chimeric immunoglobulin may be "humanized" by grafting the complementarity determining regions (CDRs), which confer binding specificity for an antigen, derived from a murine antibody into human-derived V region framework regions and human-derived constant regions. Fragments of these molecules may be generated by proteolytic digestion, or optionally, by proteolytic digestion followed by mild reduction of disulfide bonds and alkylation, or by recombinant genetic engineering techniques.

Antibodies are defined to be "immunospecific" if they specifically bind the ligand with an affinity constant, K_a, of greater than or equal to about 10^4 M^{-1}, preferably of greater than or equal to about 10^5 M^{-1}, more preferably of greater than or equal to about 10^6 M^{-1}, and still more preferably of greater than or equal to about 10^7 M^{-1}. Affinities of binding partners or antibodies can be readily determined using conventional techniques, for example, those described by Scatchard *et al.* (*Ann. N.Y. Acad. Sci. USA* 51:660, 1949) or by surface plasmon resonance (BIAcore, Biosensor, Piscataway, NJ) *See, e.g.,* Wolff *et al.*, *Cancer Res.*, 53:2560-2565, 1993).

Antibodies may generally be prepared by any of a variety of techniques known to those having ordinary skill in the art (*See, e.g.,* Harlow *et al.*, *Antibodies: A Laboratory Manual,* 1988, Cold Spring Harbor Laboratory). In one such technique, an animal is immunized with the ligand as antigen to generate polyclonal antisera. Suitable animals include rabbits, sheep, goats, pigs, cattle, and may include smaller mammalian species, such as, mice, rats, and hamsters.

An immunogen may be comprised of cells expressing the ligand, purified or partially purified ligand polypeptides or variants or fragments thereof, or ligand peptides. Ligand peptides may be generated by proteolytic cleavage or may be chemically synthesized. Peptides for immunization may be selected by analyzing the primary, secondary, or tertiary structure of the ligand according to methods know to those skilled in the art in order to determine amino acid sequences more likely to generate an antigenic response in a host animal (*See, e.g.*, Novotny, *Mol. Immunol.* 28:201-207, 1991; Berzoksky, *Science* 229:932-40, 1985).

Preparation of the immunogen may include covalent coupling of the ligand polypeptide or variant or fragment thereof, or peptide to another immunogenic protein, such as, keyhole limpet hemocyanin or bovine serum albumin. In addition, the peptide, polypeptide, or cells may be emulsified in an adjuvant (*See* Harlow *et al.*, *Antibodies: A Laboratory Manual,* 1988 Cold Spring Harbor Laboratory). In general, after the first injection, animals receive one or more booster immunizations according to a preferable schedule for the animal species. The immune response may be monitored by periodically bleeding the animal, separating the sera, and analyzing the sera in an immunoassay, such as an Ouchterlony assay, to assess the specific antibody titer. Once an antibody titer is established, the animals may be bled periodically to accumulate the polyclonal antisera. Polyclonal antibodies that bind specifically to the ligand polypeptide or peptide may then be purified from such antisera, for example, by affinity chromatography using protein A or using the ligand polypeptide or peptide coupled to a suitable solid support.

Monoclonal antibodies that specifically bind ligand polypeptides or fragments or variants thereof may be prepared, for example, using the technique of

Kohler and Milstein (*Nature*, 256:495-497, 1975; *Eur. J. Immunol.* 6:511-519, 1976) and improvements thereto. Hybridomas, which are immortal eucaryotic cell lines, may be generated that produce antibodies having the desired specificity to a the ligand polypeptide or variant or fragment thereof. An animal—for example, a rat, hamster, or preferably mouse—is immunized with the ligand immunogen prepared as described above. Lymphoid cells, most commonly, spleen cells, obtained from an immunized animal may be immortalized by fusion with a drug-sensitized myeloma cell fusion partner, preferably one that is syngeneic with the immunized animal. The spleen cells and myeloma cells may be combined for a few minutes with a membrane fusion-promoting agent, such as polyethylene glycol or a nonionic detergent, and then plated at low density on a selective medium that supports the growth of hybridoma cells, but not myeloma cells. In one embodiment, the selection media is HAT (hypoxanthine, aminopterin, thymidine). After a sufficient time, usually about 1 to 2 weeks, colonies of cells are observed. Single colonies are isolated, and antibodies produced by the cells may be tested for binding activity to the ligand polypeptide or variant or fragment thereof. Hybridomas producing antibody with high affinity and specificity for the ligand antigen are preferred. Hybridomas that produce monoclonal antibodies that specifically bind to a ligand polypeptide or variant or fragment thereof are contemplated by the present invention.

Monoclonal antibodies may be isolated from the supernatants of hybridoma cultures. An alternative method for production of a murine monoclonal antibody is to inject the hybridoma cells into the peritoneal cavity of a syngeneic mouse. The mouse produces ascites fluid containing the monoclonal antibody. Contaminants may be removed from the antibody by conventional techniques, such as chromatography, gel filtration, precipitation, or extraction.

Human monoclonal antibodies may be generated by any number of techniques. Methods include but are not limited to, Epstein Barr Virus (EBV) transformation of human peripheral blood cells (*see*, U. S. Patent No. 4,464,456), *in vitro* immunization of human B cells (*see*, *e.g.*, Boerner *et al.*, *J. Immunol.* 147:86-95, 1991), fusion of spleen cells from immunized transgenic mice carrying human

immunoglobulin genes and fusion of spleen cells from immunized transgenic mice carrying immunoglobulin genes inserted by yeast artificial chromosome (YAC) (*see, e.g.,* U. S. Patent No. 5,877,397; Bruggemann *et al.*, *Curr. Opin. Biotechnol.* 8:455-58, 1997; Jakobovits *et al.*, *Ann. N. Y. Acad. Sci.* 764:525-35, 1995), or isolation from human immunoglobulin V region phage libraries.

Chimeric antibodies and humanized antibodies for use in the present invention may be generated. A chimeric antibody has at least one constant region domain derived from a first mammalian species and at least one variable region domain derived from a second distinct mammalian species (*See, e.g.,* Morrison *et al.*, *Proc. Natl. Acad. Sci. USA*, 81:6851-55, 1984). Most commonly, a chimeric antibody may be constructed by cloning the polynucleotide sequences that encode at least one variable region domain derived from a non-human monoclonal antibody, such as the variable region derived from a murine, rat, or hamster monoclonal antibody, into a vector containing sequences that encode at least one human constant region. (S*ee, e.g.,* Shin *et al.*, *Methods Enzymol.* 178:459-76, 1989; Walls *et al.*, *Nucleic Acids Res.* 21:2921-29, 1993). The human constant region chosen may depend upon the effector functions desired for the particular antibody. Another method known in the art for generating chimeric antibodies is homologous recombination (U.S. Patent No. 5,482,856). Preferably, the vectors will be transfected into eukaryotic cells for stable expression of the chimeric antibody.

A non-human/human chimeric antibody may be further genetically engineered to create a "humanized" antibody. Such an antibody has a plurality of CDRs derived from an immunoglobulin of a non-human mammalian species, at least one human variable framework region, and at least one human immunoglobulin constant region. Humanization may yield an antibody that has decreased binding affinity when compared with the non-human monoclonal antibody or the chimeric antibody. Those having skill in the art, therefore, use one or more strategies to design humanized antibodies.

Within certain embodiments, the use of antigen-binding fragments of antibodies may be preferred. Such fragments include Fab fragments or $F(ab')_2$

fragments, which may be prepared by proteolytic digestion with papain or pepsin, respectively. The antigen binding fragments may be separated from the Fc fragments by affinity chromatography, for example, using immobilized protein A or immobilized ligand polypeptide or a variant or a fragment thereof. An alternative method to generate Fab fragments includes mild reduction of $F(ab')_2$ fragments followed by alkylation (*See, e.g.,* Weir, *Handbook of Experimental Immunology*, 1986, Blackwell Scientific, Boston).

Non-human, human, or humanized heavy chain and light chain variable regions of any of the above described Ig molecules may be constructed as single chain Fv (sFv) fragments (single chain antibodies). *See, e.g.*, Bird *et al.*, *Science* 242:423-426, 1988; Huston *et al.*, *Proc. Natl. Acad. Sci. USA* 85:5879-5883, 1988. Multi-functional fusion proteins may be generated by linking polynucleotide sequences encoding an sFv in-frame with polynucleotide sequences encoding various effector proteins. These methods are known in the art, and are disclosed, for example, in EP-B1-0318554, U.S. Patent No. 5,132,405, U.S. Patent No. 5,091,513, and U.S. Patent No. 5,476,786.

An additional method for selecting antibodies that specifically bind to a ligand polypeptide or variant or fragment thereof is by phage display (*See, e.g.*, Winter *et al.*, *Annul. Rev. Immunol.* 12:433-55, 1994; Burton *et al.*, *Adv. Immunol.* 57:191-280, 1994). Human or murine immunoglobulin variable region gene combinatorial libraries may be created in phage vectors that can be screened to select Ig fragments (Fab, Fv, sFv, or multimers thereof) that bind specifically to a ligand polypeptide or variant or fragment thereof (*See, e.g.*, U.S. Patent No. 5,223,409; Huse *et al.*, *Science* 246:1275-81, 1989; Kang *et al.*, *Proc. Natl. Acad. Sci. USA* 88:4363-66, 1991; Hoogenboom *et al.*, *J. Molec. Biol.* 227:381-388, 1992; Schlebusch *et al.*, *Hybridoma* 16:47-52, 1997 and references cited therein).

Cell Populations

As discussed above, the present invention has broad applicability to any cell type having a cell surface moiety that one is desirous of ligating. In this regard, many cell signaling events can be enhanced by the methods of the present invention.

Such methodologies can be used therapeutically in an *ex vivo* setting to activate and stimulate cells for infusion into a patient or could be used *in vivo*, to induce cell signaling events on a target cell population. However, as also noted above, the prototypic example provided herein is directed to T cells, but is in no way limited thereto.

With respect to T cells, the T cell populations resulting from the various expansion methodologies described herein may have a variety of specific phenotypic properties, depending on the conditions employed. Such phenotypic properties include enhanced expression of CD25, CD154, IFN-γ and GM-CSF, as well as altered expression of CD137, CD134, CD62L, and CD49d. The ability to differentially control the expression of these moieties may be very important. For example, higher levels of surface expression of CD154 on "tailored T cells," through contact with CD40 molecules expressed on antigen-presenting cells (such as dendritic cells, monocytes, and even leukemic B cells or lymphomas), will enhance antigen presentation and immune function. Such strategies are currently being employed by various companies to ligate CD40 via antibodies or recombinant CD40L. The approach described herein permits this same signal to be delivered in a more physiological manner, *e.g.*, by the T cell. The ability to increase IFN-γ secretion by tailoring the T cell activation (XCELLERATE) process could help promote the generation of TH1-type immune responses, important for anti-tumor and anti-viral responses. Like CD154, increased expression of GM-CSF can serve to enhance APC function, particularly through its effect on promoting the maturation of APC progenitors into more functionally competent APC, such as dendritic cells. Altering the expression of CD137 and CD134 can effect a T cell's ability to resist or be susceptible to apoptotic signals. Controlling the expression of adhesion/homing receptors, such as CD62L and/or CD49d may determine the ability of infused T cells to home to lymphoid organs, sites of infection, or tumor sites.

An additional aspect of the present invention provides a T cell population or composition that has been depleted of $CD8^+$ or $CD4^+$ cells prior to expansion. In one embodiment, $CD8^+$ cells are depleted by antibodies directed to the

$CD8^+$ marker. One of ordinary skill in the art would readily be able to identify a variety of particular methodologies for depleting a sample of $CD8^+$ or $CD4^+$ cells or conversely enriching the $CD4^+$ or $CD8^+$ cell content. With respect to enriching for $CD4^+$ cells, one aspect of the present invention is focused on the identification of an extremely robust CD154 expression profile upon stimulation of T cell populations wherein T_C ($CD8^+$) cells have been depleted. As indicated above, CD154 is an important immunomodulating molecule whose expression is extremely beneficial in amplifying the immune response. Accordingly an increase in CD154 expression is likely to lead to more efficacious T cell compositions.

An additional aspect of the present invention provides a T cell population or composition that has been depleted or enriched for populations of cells expressing a variety of markers, such as CD62L, CD45RA or CD45RO, cytokines (*e.g.* IL-2, IFN-γ, IL-4, IL-10), cytokine receptors (*e.g.* CD25), perforin, adhesion molecules (*e.g.* VLA-1, VLA-2, VLA-4, LPAM-1, LFA-1), and/or homing molecules (*e.g.* L-Selectin), prior to expansion. In one embodiment, cells expressing any of these markers are depleted or positively selected by antibodies or other ligands/binding agents directed to the marker. One of ordinary skill in the art would readily be able to identify a variety of particular methodologies for depleting or positively selecting for a sample of cells expressing a desired marker.

The present invention further provides methods for activating and expanding regulatory T cells. Regulatory T cells can be generated using art-recognized techniques as described for example, in Woo, *et al.*, J Immunol. 2002 May 1;168(9):4272-6; Shevach, E.M., Annu. Rev. Immunol. 2000, 18:423; Stephens, *et al.*, Eur. J. Immunol. 2001, 31:1247; Salomon, et al, Immunity 2000, 12:431; and Sakaguchi, et al., Immunol. Rev. 2001, 182:18.

With respect to the prototypic example of T cells, the methodologies described herein can be used to selectively expand a population of $CD28^+$, $CD4^+$, $CD8^+$, $CD45RA^+$, or $CD45RO^+$ T cells for use in the treatment of infectious diseases, cancer, and immunotherapy. As a result, a phenotypically unique population of T cells, which is polyclonal with respect to antigen reactivity, but essentially homogeneous with

respect to either $CD4^+$ or $CD8^+$ can be produced. In addition, the method allows for the expansion of a population of T cells in numbers sufficient to reconstitute an individual's total $CD4^+$ or $CD8^+$ T cell population (the population of lymphocytes in an individual is approximately 3-5 X 10^{11}). The resulting T cell population can also be genetically transduced and used for immunotherapy or can be used in methods of *in vitro* analyses of infectious agents. For example, a population of tumor-infiltrating lymphocytes can be obtained from an individual afflicted with cancer and the T cells stimulated to proliferate to sufficient numbers. The resulting T cell population can be genetically transduced to express tumor necrosis factor (TNF) or other proteins (for example, any number of cytokines, inhibitors of apoptosis (*e.g.* Bcl-2), genes that protect cells from HIV infection such as RevM10 or intrakines, and the like, targeting molecules, adhesion and/or homing molecules and any variety of antibodies or fragments thereof (*e.g.* Scfv)) and given to the individual.

The phenotypic properties of T cell populations of the present invention can be monitored by a variety of methods including standard flow cytometry methods and ELISA methods known by those skilled in the art.

Methods of Use

In addition to the methods described above, cells stimulated and/or activated by the methods herein described may be utilized in a variety of contexts. The cells may be administered to patients who have decreased hematologic function resulting from a variety of diseases, treatments, or a combination thereof, to accelerate hematologic recovery. In particular, the cells of the present invention may be used to accelerate hematologic recovery following treatments for a variety of cancers. The cancer may be any one of melanoma, non-Hodgkin's lymphoma, Hodgkin's disease, leukemia, plasmocytoma, sarcoma, glioma, thymoma, breast cancer, prostate cancer, colo-rectal cancer, kidney cancer, renal cell carcinoma, pancreatic cancer, nasopharyngeal carcinoma, esophageal cancer, brain cancer, lung cancer, ovarian cancer, cervical cancer, multiple myeloma, heptocellular carcinoma, acute lymphoblastic leukemia (ALL), acute myelogenous leukemia (AML), chronic

myelogenous leukemia (CML), and chronic lymphocytic leukemia (CLL). In one embodiment, the cancer is B-cell lymphocytic leukemia. In a further embodiment, the cancer is multiple myeloma.

In certain embodiments of the present invention, the cells of the present invention are administered to a patient following treatment with an agent such as myeloablative (high dose) chemotherapy, chemotherapy, radiation, immunosuppressive agents, such as cyclosporine, azathioprine, methotrexate, mycophenolate, and FK506, antibodies, or other immunoablative agents such as CAMPATH, anti-CD3 antibodies, cyclophosphamide, fludarabine, cyclosporine, FK506, rapamycin, mycophenolic acid, steroids, FR901228, and irradiation. These drugs inhibit either the calcium dependent phosphatase calcineurin (cyclosporine and FK506) or inhibit the p70S6 kinase that is important for growth factor induced signaling (rapamycin). (Liu et al., Cell 66:807-815, 1991; Henderson et al., Immun. 73:316-321, 1991; Bierer et al., Curr. Opin. Immun. 5:763-773, 1993; Isoniemi (supra)).

In certain embodiments, the cells of the present invention are administered following or in conjunction with transplantation such as stem cell transplantation and any treatment associated therewith, such as treatment with one or more of the agents described herein and/or treatment with other agents such as NEUPOGEN and LEUKINE (recombinant forms of G-CSF and GM-CSF). In one embodiment, the cells of the present invention are administered following myeloablative chemotherapy and autologous stem cell transplant wherein the patient is experiencing neutropenia. In certain embodiments, the cells of the present invention are administered to prevent neutropenia, to minimize the number of days that neutropenia occurs and/or to reduce the risk of infections associated with decreased hematologic function such as neutropenia. In other embodiments, the cells of the present invention are administered to patients with low platelet counts associated with cancers and/or treatments described herein. In this regard, the cells of the present invention may be used to reduce the risk of bleeding associated with low platelet counts.

The cells of the present invention can be administered to any individual with hematologic disorders, such as individuals who have undergone any number of

surgeries, burn patients, trauma patients, patients with bone marrow dysfunction or other disorders of hematopoiesis.

The cells of the present invention can be administered alone, or in conjunction with (prior to, at the same time, or following) the treatments described herein, including treatment with cytokines and/or growth factors including but not limited to IL-2, granulocyte colony-stimulating factor (G-CSF), macrophage colony stimulating factor (M-CSF), granulocyte-macrophage colony-stimulating factor (GM-CSF), interleukin 4 (IL-4), IL-13, interleukin 1α (IL-1α) and β (IL-1β), tumor necrosis factor alpha (TNF-α), interleukin 3 (IL-3), stem cell factor (SCF), interleukin 6 (IL-6), and Flt3-L.

In certain embodiments, the cells described herein are administered with (prior to, at the same time as, or following) other cells, such as stem cells, dendritic cells, or neutrophils. In other embodiments, the cells described herein are administered with (prior to, at the same time as, or following) platelets. In further embodiments, the cells of the present invention are administered with monoclonal antibodies, any of a variety of antibiotics known in the art, or anti-fungal therapies known to the skilled artisan.

The methods for stimulating and expanding a population of antigen specific T cells are useful in therapeutic situations where it is desirable to up-regulate an immune response (*e.g.*, induce a response or enhance an existing response) upon administration of the T cells to a subject. For example, the method can be used to enhance a T cell response against tumor-associated antigens. Tumor cells from a subject typically express tumor-associated antigens but may be unable to stimulate a co-stimulatory signal in T cells (*e.g.*, because they lacks expression of co-stimulatory molecules). Thus, tumor cells can be contacted with T cells from the subject *in vitro* and antigen specific T cells expanded according to the method of the invention and the T cells returned to the subject.

Accordingly, in one embodiment malignancies such as non-Hodgkins Lymphoma (NHL) and B-cell chronic lymphocytic leukemia (B-CLL) can be treated. While initial studies using expanded T cells have been tested in NHL, (see Liebowitz *et*

al., Curr. Opin. Onc. 10:533-541, 1998), the T cell populations of the present invention offer unique phenotypic characteristics that can dramatically enhance the success of immunotherapy by providing increased engraftment (likely supplied by stimulation of the CD28 signal) and reactivity. However, patients with B-CLL present special difficulties, including low relative T cell numbers with high leukemic cell burden in the peripheral blood, accompanied by a general T cell immunosuppression. The T cell populations of the present invention can provide dramatically improved efficacy in treating this disease and especially when combined with stem cell transplantation therapy. Accordingly, increasing T cell function and anti-CLL T cell activity with anti-CD3 x anti-CD28 co-immobilized beads would be beneficial.

For example, given that deficient expression of CD154, the ligand for CD40, on T cells of B-CLL patients has been cited as a major immunological defect of the disease, the T cell populations of the present invention, which may provide sustained high levels of CD154 expression upon re-infusion, could aid in its treatment. Investigators report that in CLL the capability of a patient's T cells' to express CD154 is defective as well as the capability of the leukemic B-cells to express CD80 and CD86. The failure of leukemic B-cells in CLL to adequately express the ligands for CD28, could result in failure to fully activate tumor-responsive T cells and, therefore, may represent the mechanism underlying the T cells' apparent state of tolerance. Studies in which CD40 is engaged on CLL B cells, either via soluble anti-CD40 antibodies or via CD154-transduced leukemic B-cells, appears to correct the defect in CD80 and CD86 expression and up-regulates MHC surface expression. Kato *et al., J. Clin. Invest. 101*:1133-1141, 1998; Ranheim and Kipps, *J. Exp. Med. 177*:925-935, 1993. Cells treated in this way were able to stimulate specific T cell anti-tumor responses.

With the enhanced expression of CD154 on the surface of the T cell population of the present invention such T cells would be expected to interact with autologous B-CLL cells, and would thus increase that tumor's immunogenicity by driving up expression of MHC, CD80, and CD86. This, in turn, should lead to a strong anti-tumor response. Further, one of ordinary skill in the art would readily understand

that treatment of a patient with *ex vivo* expanded T cells of the present invention may be combined with traditional cancer therapies such as chemotherapy. In this regard, for example, a patient may be treated with an agent such as Fludarabine or Campath (Berlex Laboratories, Montville, NJ, USA), followed by infusion with T cell populations of the present invention or both.

Alternatively, T cells can be stimulated and expanded as described herein to induce or enhance responsiveness to pathogenic agents, such as viruses (*e.g.*, human immunodeficiency virus), bacteria, parasites and fungi.

The invention further provides methods to selectively expand a specific subpopulation of T cells from a mixed population of T cells. In particular, the invention provides specifically enriched populations of T cells that have much higher ratio of $CD4^+$ and $CD8^+$ double positive T cells.

Another embodiment of the invention, provides a method for selectively expanding a population of T_{H1} cells from a population of $CD4^+$ T cells. In this method, $CD4^+$ T cells are co-stimulated with an anti-CD28 antibody, such as the monoclonal antibody 9.3, inducing secretion of T_{H1}-specific cytokines, including IFN-γ, resulting in enrichment of T_{H1} cells over T_{H2} cells.

The observation herein that phenotypic traits of activated T cells vary over time during the expansion process, combined with the fact that T cells have been demonstrated to be activated within a few hours (Iezzi *et al.*, Immunity 8:89-95, 1998). Accordingly, in combination with the methodologies herein described, this provides the ability to expand a tailor made subset of a T cell population in a short period of time. In one embodiment, this technique can be utilized at the bedside of a subject, in an outpatient modality, or at a subject's home, similar to the use of kidney dialysis. For example, a method or device wherein T cells are incubated in contact with activation signals (*e.g.*, anti-CD3 and anti-CD28 antibodies, and the like) and returned to the patient immediately in a continuous flow or after a few hour expansion period. In one aspect, such techniques of expansion could use isolated chambers with filter components, such that 3x28 beads or similarly coated microparticles are mixed with a continuous flow of blood/ concentrated cells. In another embodiment, solid surfaces

within an apparatus may be coated or conjugated directly (including covalently) or indirectly (*e.g.*, streptavidin/biotin and the like) with antibodies or other components to stimulate T cell activation and expansion. For example, a continuous fluid path from the patient through a blood/cell collection device and/or a disposable device containing two or more immobilized antibodies (*e.g.,* anti-CD3 and anti-CD28) or other components to stimulate receptors required for T cell activation prior to cells returning to the subject can be utilized (immobilized on plastic surfaces or upon separable microparticles). Such a system could involve a leukapheresis instrument with a disposable set sterile docked to the existing manufacturers disposable set, or be an adaptation to the manufacturer's disposable set (*e.g.*, the surface platform on which the antibodies/activation components are immobilized/contained is within the bag/container for collection of peripheral blood mononuclear cells during apheresis). Further, the solid surface/surface platform may be a part of a removal insert which is inserted into one of the device chambers or physically present within one of the disposable components. In another embodiment of the continuous flow aspect discussed above, the system may comprise contacting the cells with the activating components at room temperature or at physiologic temperature using a chamber within a blood collection device or an incubation chamber set up in series with the flow path to the patient.

In another example, blood is drawn into a stand-alone disposable device directly from the patient that contains two or more immobilized antibodies (*e.g.,* anti-CD3 and anti-CD28) or other components to stimulate receptors required for T cell activation prior to the cells being administered to the subject (*e.g.,* immobilized on plastic surfaces or upon separable microparticles). In one embodiment, the disposable device may comprise a container (*e.g.*, a plastic bag, or flask) with appropriate tubing connections suitable for combining/docking with syringes and sterile docking devices. This device will contain a solid surface for immobilization of T cell activation components (*e.g.,* anti-CD3 and anti-CD28 antibodies); these may be the surfaces of the container itself or an insert and will typically be a flat surface, an etched flat surface, an irregular surface, a porous pad, fiber, clinically acceptable/safe ferro-fluid, beads, etc.). Additionally when using the stand-alone device, the subject can remain connected to

the device, or the device can be separable from the patient. Further, the device may be utilized at room temperature or incubated at physiologic temperature using a portable incubator.

As devices and methods for collecting and processing blood and blood products are well known, one of skill in the art would readily recognize that given the teachings provided herein, that a variety of devices that fulfill the needs set forth above may be readily designed or existing devices modified. Accordingly, as such devices and methods are not limited by the specific embodiments set forth herein, but would include any device or methodology capable of maintaining sterility and which maintains blood in a fluid form in which complement activation is reduced and wherein components necessary for T cell activation (*e.g.*, anti-CD3 and anti-CD28 antibodies or ligands thereto) may be immobilized or separated from the blood or blood product prior to administration to the subject. Further, as those of ordinary skill in the art can readily appreciate a variety of blood products can be utilized in conjunction with the devices and methods described herein. For example the methods and devices could be used to provide rapid activation of T cells from cryopreserved whole blood, peripheral blood mononuclear cells, other cyropreserved blood-derived cells, or cryopreserved T cell lines upon thaw and prior to subject administration. In another example, the methods and devices can be used to boost the activity of a previously *ex vivo* expanded T cell product or T cell line prior to administration to the subject, thus providing a highly activated T cell product. Lastly, as will be readily appreciated the methods and devices above may be utilized for autologous or allogeneic cell therapy simultaneously with the subject and donor.

The methods of the present invention may also be utilized with vaccines to enhance reactivity of the antigen and enhance *in vivo* effect. Further, given that T cells expanded by the present invention have a relatively long half-life in the body, these cells could act as perfect vehicles for gene therapy, by carrying a desired nucleic acid sequence of interest and potentially homing to sites of cancer, disease, or infection. Accordingly, the cells expanded by the present invention may be delivered to a patient in combination with a vaccine, one or more cytokines, one or more therapeutic

antibodies, etc. Virtually any therapy that would benefit by a more robust T cell population is within the context of the methods of use described herein.

Pharmaceutical Compositions

Target cell populations, such as T cell populations of the present invention may be administered either alone, or as a pharmaceutical composition in combination with diluents and/or with other components such as IL-2 or other cytokines or cell populations. Briefly, pharmaceutical compositions of the present invention may comprise a target cell population as described herein, in combination with one or more pharmaceutically or physiologically acceptable carriers, diluents or excipients. Such compositions may comprise buffers such as neutral buffered saline, phosphate buffered saline and the like; carbohydrates such as glucose, mannose, sucrose or dextrans, mannitol; proteins; polypeptides or amino acids such as glycine; antioxidants; chelating agents such as EDTA or glutathione; adjuvants (*e.g.*, aluminum hydroxide); and preservatives. Compositions of the present invention are preferably formulated for intravenous administration.

Pharmaceutical compositions of the present invention may be administered in a manner appropriate to the disease to be treated (or prevented). The quantity and frequency of administration will be determined by such factors as the condition of the patient, and the type and severity of the patient's disease, although appropriate dosages may be determined by clinical trials.

The immune response induced in a subject by administering T cells stimulated and activated using the methods described herein, or other methods known in the art wherein T cells are stimulated and expanded to therapeutic levels, may include cellular immune responses mediated by cytotoxic T cells, capable of killing tumor and infected cells, regulatory T cells, helper T cell responses, NK cells, and the like. Humoral immune responses, mediated primarily by helper T cells capable of activating B cells thus leading to antibody production, may also be induced. A variety of techniques may be used for analyzing the type of immune responses induced by the

compositions of the present invention, which are well described in the art; *e.g.*, Coligan et al. Current Protocols in Immunology, John Wiley & Sons Inc. (1994).

When "an immunologically effective amount", "an anti-tumor effective amount", "an tumor-inhibiting effective amount", or "therapeutic amount" is indicated, the precise amount of the compositions of the present invention to be administered can be determined by a physician with consideration of individual differences in age, weight, tumor size, extent of infection or metastasis, and condition of the patient (subject). It can generally be stated that a pharmaceutical composition comprising the T cells described herein may be administered at a dosage of 10^4 to 10^7 cells/kg body weight, preferably 10^5 to 10^6 cells/kg body weight, including all integer values within those ranges. T cell compositions may also be administered multiple times at these dosages. The cells can be administered by using infusion techniques that are commonly known in immunotherapy (see, *e.g.*, Rosenberg et al., New Eng. J. of Med. 319:1676, 1988). The optimal dosage and treatment regime for a particular patient can readily be determined by one skilled in the art of medicine by monitoring the patient for signs of disease and adjusting the treatment accordingly.

Typically, in adoptive immunotherapy studies, antigen-specific T cells are administered approximately at 2 X 10^9 to 2 X 10^{11} cells to the patient. (See, *e.g.*, U.S. Pat. No. 5,057,423). In some aspects of the present invention, particularly in the use of allogeneic or xenogeneic cells, lower numbers of cells, in the range of 10^6/kilogram (10^6-10^{11} per patient) may be administered. In certain embodiments, T cells are administered at 1 X10^5, 1 X 10^6, 1 X 10^7, 1 X 10^8, 2 X 10^8, 2 X 10^9, 1 X 10^{10}, 2 X 10^{10}, 1 X 10^{11}, 5 X 10^{11}, or 1 X 10^{12} cells to the subject. T cell compositions may be administered multiple times at dosages within these ranges. The cells may be autologous or heterologous to the patient undergoing therapy. If desired, the treatment may also include administration of mitogens (*e.g.*, PHA) or lymphokines, cytokines, and/or chemokines (*e.g.*, GM-CSF, IL-4, IL-13, Flt3-L, RANTES, MIP1α, etc.) as described herein to enhance induction of the immune response.

The administration of the subject compositions may be carried out in any convenient manner, including by aerosol inhalation, injection, ingestion, transfusion,

implantation or transplantation. The compositions described herein may be administered to a patient subcutaneously, intradermally, intratumorally, intranodally, intramdullary, intramuscularly, by intravenous (i.v.) injection, or intraperitoneally. In one embodiment, the T cell compositions of the present invention are administered to a patient by intradermal or subcutaneous injection. In another embodiment, the T cell compositions of the present invention are preferably administered by *i.v.* injection. The compositions of T cells may be injected directly into a tumor, lymph node, or site of infection.

In yet another embodiment, the pharmaceutical composition can be delivered in a controlled release system. In one embodiment, a pump may be used (see Langer, 1990, Science 249:1527-1533; Sefton 1987, CRC Crit. Ref. Biomed. Eng. 14:201; Buchwald et al., 1980; Surgery 88:507; Saudek et al., 1989, N. Engl. J. Med. 321:574). In another embodiment, polymeric materials can be used (see Medical Applications of Controlled Release, 1974, Langer and Wise (eds.), CRC Pres., Boca Raton, Fla.; Controlled Drug Bioavailability, Drug Product Design and Performance, 1984, Smolen and Ball (eds.), Wiley, New York; Ranger and Peppas, 1983; J. Macromol. Sci. Rev. Macromol. Chem. 23:61; see also Levy et al., 1985, Science 228:190; During et al., 1989, Ann. Neurol. 25:351; Howard et al., 1989, J. Neurosurg. 71:105). In yet another embodiment, a controlled release system can be placed in proximity of the therapeutic target, thus requiring only a fraction of the systemic dose (see, *e.g.*, Medical Applications of Controlled Release, 1984, Langer and Wise (eds.), CRC Pres., Boca Raton, Fla., vol. 2, pp. 115-138).

The T cell compositions of the present invention may also be administered using any number of matrices. Matrices have been utilized for a number of years within the context of tissue engineering (see, *e.g.*, Principles of Tissue Engineering (Lanza, Langer, and Chick (eds.)), 1997. The present invention utilizes such matrices within the novel context of acting as an artificial lymphoid organ to support, maintain, or modulate the immune system, typically through modulation of T cells. Accordingly, the present invention can utilize those matrix compositions and formulations which have demonstrated utility in tissue engineering. Accordingly, the

type of matrix that may be used in the compositions, devices and methods of the invention is virtually limitless and may include both biological and synthetic matrices. In one particular example, the compositions and devices set forth by U.S. Patent Nos: 5,980,889; 5,913,998; 5,902,745; 5,843,069; 5,787,900; or 5,626,561 are utilized. Matrices comprise features commonly associated with being biocompatible when administered to a mammalian host. Matrices may be formed from both natural or synthetic materials. The matrices may be non-biodegradable in instances where it is desirable to leave permanent structures or removable structures in the body of an animal, such as an implant; or biodegradable. The matrices may take the form of sponges, implants, tubes, telfa pads, fibers, hollow fibers, lyophilized components, gels, powders, porous compositions, or nanoparticles. In addition, matrices can be designed to allow for sustained release seeded cells or produced cytokine or other active agent. In certain embodiments, the matrix of the present invention is flexible and elastic, and may be described as a semisolid scaffold that is permeable to substances such as inorganic salts, aqueous fluids and dissolved gaseous agents including oxygen.

A matrix is used herein as an example of a biocompatible substance. However, the current invention is not limited to matrices and thus, wherever the term matrix or matrices appears these terms should be read to include devices and other substances which allow for cellular retention or cellular traversal, are biocompatible, and are capable of allowing traversal of macromolecules either directly through the substance such that the substance itself is a semi-permeable membrane or used in conjunction with a particular semi-permeable substance.

In certain embodiments of the present invention, cells activated and expanded using the methods described herein, or using other methods known in the art where T cells are expanded to therapeutic levels, are administered to a patient in conjunction with (*e.g.* before, simulataneously or following) any number of relevant treatment modalities, including but not limited to treatment with agents such as antiviral agents, chemotherapy, radiation, immunosuppressive agents, such as cyclosporin, azathioprine, methotrexate, mycophenolate, and FK506, antibodies, or other immunoablative agents such as CAMPATH, anti-CD3 antibodies or other antibody

therapies, cytoxin, fludaribine, cyclosporin, FK506, rapamycin, mycophenolic acid, steroids, FR901228, cytokines, and irradiation. These drugs inhibit either the calcium dependent phosphatase calcineurin (cyclosporine and FK506) or inhibit the p70S6 kinase that is important for growth factor induced signaling (rapamycin). (Liu et al., Cell 66:807-815, 1991; Henderson et al., Immun. 73:316-321, 1991; Bierer et al., Curr. Opin. Immun. 5:763-773, 1993; Isoniemi (supra)). In a further embodiment, the cell compositions of the present invention are administered to a patient in conjunction with (*e.g.* before, simulataneously or following) bone marrow transplantation, T cell ablative therapy using either chemotherapy agents such as, fludarabine, external-beam radiation therapy (XRT), cyclophosphamide, or antibodies such as OKT3 or CAMPATH. In another embodiment, the cell compositions of the present invention are administered following B-cell ablative therapy such as agents that react with CD20, *e.g.* Rituxan. For example, in one embodiment, subjects may undergo standard treatment with high dose chemotherapy followed by peripheral blood stem cell transplantation. In certain embodiments, following the transplant, subjects receive an infusion of the expanded immune cells of the present invention. In an additional embodiment, expanded cells are administered before or following surgery.

The dosage of the above treatments to be administered to a patient will vary with the precise nature of the condition being treated and the recipient of the treatment. The scaling of dosages for human administration can be performed according to art-accepted practices. The dose for CAMPATH, for example, will generally be in the range 1 to about 100 mg for an adult patient, usually administered daily for a period between 1 and 30 days. The preferred daily dose is 1 to 10 mg per day although in some instances larger doses of up to 40 mg per day may be used (described in U.S. Patent No. 6,120,766).

In a further embodiment, the cell compositions comprising T cells stimulated and activated using the methods described herein, or other methods known in the art wherein T cells are stimulated and expanded to therapeutic levels, are administered to a patient in conjunction with allogeneic stem cell transplantation (such as in a mini-transplant setting) or organ transplantation. Without being bound by

theory, such T cells may enhance and promote engraftment and anti-tumor effects. These T cells may have enhanced stem cell graft promoting effects and anti-tumor effects that allow a much reduced and less toxic transplant conditioning regimen to be utilized.

All references referred to within the text are hereby incorporated by reference in their entirety. Moreover, all numerical ranges utilized herein explicitly include all integer values within the range and selection of specific numerical values within the range is contemplated depending on the particular use. Further, the following examples are offered by way of illustration, and not by way of limitation.

EXAMPLES

EXAMPLE 1

T CELL STIMULATION

Generally, T cell stimulation, activation and expansion is carried out as described in U.S. Patent Applications numbers 10/350305, 10/187,467, 10/133,236, 09/960,264, and 09/794,230.

In certain experiments described herein, the process referred to as XCELLERATE I™ was utilized. In brief, in this process, the XCELLERATED™ T cells are manufactured from a peripheral blood mononuclear cell (PBMC) apheresis product. After collection from the patient at the clinical site, the PBMC apheresis are washed and then incubated with "uncoated" DYNABEADS® M-450 Epoxy T. During this time phagocytic cells such as monocytes ingest the beads. After the incubation, the cells and beads are processed over a MaxSep Magnetic Separator in order to remove the beads and any monocytic/phagocytic cells that are attached to the beads. Following this monocyte-depletion step, a volume containing a total of 5×10^8 $CD3^+$ T cells is taken and set-up with 1.5×10^9 DYNABEADS® M-450 CD3/CD28 T to initiate the

XCELLERATE™ process (approx. 3:1 beads to T cells). The mixture of cells and DYNABEADS® M-450 CD3/CD28 T are then incubated at 37°C, 5% CO_2 for approximately 8 days to generate XCELLERATED T cells for a first infusion. The remaining monocyte-depleted PBMC are cryopreserved until a second or further cell product expansion (approximately 21 days later) at which time they are thawed, washed and then a volume containing a total of 5 x 10^8 $CD3^+$ T cells is taken and set-up with 1.5 x 10^9 DYNABEADS® M-450 CD3/CD28 T to initiate the XCELLERATE Process for a second infusion. During the incubation period of ≈8 days at 37°C, 5% CO_2, the $CD3^+$ T cells activate and expand. The anti-CD3 mAb used is BC3 (XR-CD3; Fred Hutchinson Cancer Research Center, Seattle, WA), and the anti-CD28 mAb (B-T3, XR-CD28) is obtained from Diaclone, Besançon, France .

With a modified process referred to as XCELLERATE II™ the process described above was utilized with some modifications in which no separate monocyte depletion step was utilized and in certain processes the cells were frozen prior to initial contact with beads and further concentration and stimulation were performed. (See Figures 5A and 5B). In one version of this process T cells were obtained from the circulating blood of a donor or patient by apheresis. Components of an apheresis product typically include lymphocytes, monocytes, granulocytes, B cells, other nucleated cells (white blood cells), red blood cells, and platelets. A typical apheresis product contains 1 – 2 x10^{10} nucleated cells. The cells are washed with calcium-free, magnesium-free phosphate buffered saline to remove plasma proteins and platelets. The washing step was performed by centrifuging the cells and removing the supernatant fluid, which is then replaced by PBS. The process was accomplished using a semi-automated "flow through" centrifuge (COBE 2991 System, Baxter). The cells are maintained in a closed system as they are processed.

The cells may be further processed by depleting the non-binding cells, including monocytes, (enriched for activated cells) and then continuing with the stimulation. Alternatively, the washed cells can be frozen, stored, and processed later, which is demonstrated herein to increase robustness of proliferation as well as depleting granulocytes. In one example, to freeze the cells, a 35 ml suspension of cells is placed

in a 250 ml Cryocyte freezing bag along with 35 ml of the freezing solution. The 35 ml cell suspension typically contains $3.5x10^9$ to $5.0x10^9$ cells in PBS. An equal volume of freezing solution (20% DMSO and 8% human serum albumin in PBS) is added. The cells are at a final concentration of $50x10^6$ cells/ml. The Cryocyte bag may contain volumes in the range of 30 – 70 ml, and the cell concentration can range from 10 to $200x10^6$ cells/ml. Once the Cryocyte bag is filled with cells and freezing solution, the bag is placed in a controlled rate freezer and the cells are frozen at 1°C/minute down to -80°C. The frozen cells are then placed in a liquid nitrogen storage system until needed.

The cells are removed from the liquid nitrogen storage system and are thawed at 37 ° C. To remove DMSO, the thawed cells are then washed with calcium-free, magnesium-free PBS on the COBE 2991 System. The washed cells are then passed through an 80 micron mesh filter.

The thawed cells, approximately $0.5x10^9$ $CD3^+$ cells, are placed in a plastic 1L Lifecell bag that contains 100 ml of calcium-free, magnesium-free PBS. The PBS contains 1% - 5% human serum. $1.5x10^9$ 3x28 beads (DYNABEADS® M-450 CD3/CD28 T) are also placed in the bag with the cells (3:1 DYNABEADS M-450 CD3/CD28 T:$CD3^+$ T cells). The beads and cells are mixed at room temperature at ~ 1 RPM (end-over-end rotation) for about 30 minutes. The bag containing the beads and cells is placed on the MaxSep Magnetic Separator (Nexell Therapeutics, Irvine, CAb. Between the bag and the MaxSep, a plastic spacer (approximately 6 mm thick) is placed. (To increase the magnetic strength the spacer is removed.) The beads and any cells attached to beads are retained on the magnet while the PBS and unbound cells are pumped away.

The 3x28 beads and concentrated cells bound to the beads are rinsed with cell culture media (1 liter containing X-Vivo 15, BioWhittaker; with 50 ml heat inactivated pooled human serum, 20 ml 1M Hepes, 10 ml 200 mM L-glutamine with or without about 100,000 I.U. IL-2) into a 3L Lifecell culture bag. After transferring the 3x28 beads and positively selected cells into the Lifecell bag, culture media is added until the bag contains 1000 ml. The bag containing the cells is placed in an incubator (37°C and 5% CO_2) and cells are allowed to expand.

Cells were split 1 to 4 on each of days 3 and 5. T cell activation and proliferation were measured by harvesting cells after 3 days and 8 days in culture. Activation of T cells was assessed by measuring cell size, the level of cell surface marker expression, particularly the expression of CD25 and CD154 on day 3 of culture. On day 8 cells were allowed to flow under gravity (approx. 150 ml/min) over the MaxSep magnet to remove the magnetic particles and the cells are washed and concentrated using the COBE device noted above and resuspended in a balanced electrolyte solution suitable for intravenous administration, such as Plasma-Lyte A® (Baxter-Healthcare).

As described within the specification XCELLERATE I™ refers to conditions similar to that above, except that stimulation and concentration were not performed and monocyte depletion was performed prior to stimulation.

Both XCELLERATE I™ and II™ processes were performed and T cell proliferation was measured after 8 days in culture. The yield of expanded T cells was greater when $CD3^+$ cells were concentrated prior to cell culture. (See Table 1). In addition, the cell population had greater than 90% $CD3^+$ cells.

Table 1. T cell Yield Expansion at Day 8

Experiment	No $CD3^+$ Concentration (XCELLERATE I™)	$CD3^+$ Concentration (XCELLERATE II™)
NDa079	33×10^9	36×10^9
NDa081	38×10^9	42×10^9
NDa082	28×10^9	38×10^9
Average	$33 \pm 5 \times 10^9$	$39 \pm 3 \times 10^9$

Further experiments were performed in this regard and depict total number of expanded cells as well as the fold expansion of nine batches of cells stimulated without $CD3^+$ concentration and five batches of cells stimulated with $CD3^+$ concentration. (See Figures 1 and 2).

Concentration of the cells by application of a magnetic force prior to culture effectively increases the purity of the $CD3^+$ cells as well as increasing CD154 levels. (Table 2, Figures 3 and 4 depict CD154 levels graphically). Furthermore, comparison of T cell proliferation where populations of T cells were exposed to magnets of differing strengths showed that exposure to a stronger magnet resulted in greater yield of $CD3^+$ cells. (Table 2.)

Table 2. Comparison of T cell Proliferation and Cell Surface Markers after Concentration Using Weak and Strong Magnets

Experiment	Magnet	Day	CD3%	Size (FSC)	CD25 (MFI)	CD154 (MFI)	CD3# x 10^9
NDa087							
Pre-Selection		0	47%	318	8	4	0.5
Post-Selection	Weak	0	56%				0.37
Post-Selection	Strong	0	61%				0.35
No Selection	None	3		533	758	19	
Post-Selection	Weak	3	90%	570	846	41	
Post-Selection	Strong	3	92%	558	1006	45	
Post-Culture	None						
Post-Culture	Weak	8	92%	412	110	9	17.7
	Strong	8	93%	413	89	7	37.8
NDa089							
Pre-Selection		0	44%	312	6	4	0.5
Post-Selection	Weak	0	46%				0.39
Post-Selection	Strong	0	55%				0.3
Post-Selection	Weak	3	83%	589	685	67	
Post-Selection	Strong	3	83%	600	720	115	
Post-Culture	Weak	8	89%	409	58	18	25.3
	Strong	8	87%	371	65	13	42.1

Table 2. (continued)

Experiment	Magnet	CD25 on Day 0	CD25 on Day 3	CD154 on Day 0	CD154 on Day 3	CD3 Cell # On Day 8
		(MFI)	(MFI)	(MFI)	(MFI)	x 10^9
NDa087						
No Selection	None	8	758	4	19	31
Selection	Weak	8	846	4	41	18
Selection	Strong	8	1006	4	45	38
NDa089						
No Selection	None	6	309	4	12	26
Selection	Weak	6	685	4	67	25
Selection	Strong	6	720	4	115	42

Five additional experiments were performed comparing the process of XCELLERATE I™ to that of XCELLERATE II™. For the cells activated and culture-expanded according to the two processes, cell activation markers (cell size, CD25 expression, and CD154 expression) on days 3 and 8 of culture are shown below in Table 3 and in Figures 6-7.

Table 3: Cell Activation Markers on Day 3

Experiment Number (Donor)	Process	Cell Size (FSC)		CD25 (MFI)		CD154 (MFI)	
		Day 0	Day 3	Day 0	Day 3	Day 0	Day 3
NDa104 (PC071)	XCELLERATEI	282	526	7	625	5	50
	XCELLERATE II	315	531	7	750	5	162
NDa107 (PC074)	XCELLERATE I	243	578	5	287	4	23
	XCELLERATE II	272	587	6	311	5	120
NDa110 (PC076)	XCELLERATE I	262	588	6	497	4	59
	XCELLERATE II	284	615	6	580	5	197
NDa113	XCELLERATE I	271	662	5	726	4	54

Experiment Number (Donor)	Process	Cell Size (FSC)		CD25 (MFI)		CD154 (MFI)	
		Day 0	Day 3	Day 0	Day 3	Day 0	Day 3
(PC060)	XCELLERATE II	291	660	6	741	5	177
NDa115 (PC073)	XCELLERATE I	253	560	6	202	6	25
	XCELLERATE II	252	582	6	448	6	83
Average ± Std Dev	XCELLERATE I	**262** ± 15	**583** ± 50	**6** ± 1	**467** ± 221	**5** ± 1	**42** ± 17
	XCELLERATE II	**283** ± 23	**595** ± 47	**6** ± 1	**566** ± 189	**5** ± 1	**148** ± 17

All cultures in Table 3 were initiated with cells that were frozen/thawed.

The data in Table 3 and Figures 6-7 show that the XCELLERATE II™ process generated cells whose cell size and CD25 expression activation markers on day 3 were on average similar, but typically higher and continued to be higher following stimulation. However, the CD154 activation marker on day 3 for T cells from the XCELLERATE II™ process was much greater than for those of T cells from the XCELLERATE I™ process. Further, as demonstrated above, the XCELLERATE II™ process generated CD25 and CD154 levels that were consistently higher per donor than other methods.

The expression of CD154 on Day 3 of the XCELLERATE II™ process is actually much higher than for XCELLERATE I™. This observation suggests that the T cells are in a higher state of activation during the XCELLERATE II™ process than in the XCELLERATE I™ process. It is predicted that this may translate into a more effective product when administered *in vivo*.

$CD3^+$ Cell Purity, CD4 Cell/CD8 cell ratio, and cell viability on Day 3 of culture were also determined for five patient samples. The phenotype and viability of cells used subjected to the XCELLERATE I™ process and the XCELLERATE II™ process are shown below in Table 4 as measured by Flow Cytometry or Trypan blue staining.

Table 4

NDa #	Day 0 $CD3^+$ Cell Purity (%)*	Day 0 Cell Viability (%)	Day 0 CD4:CD8 ratio$^{\Psi}$	Day 3 $CD3^+$ Cell Purity (%)	Day 3 Cell Viability (%)	Day 3 CD4:CD8 ratio
103 XCELLERATE I	70	92	1.91	79	82	1.3
103 XCELLERATE II	85	99	2.3	91	95	2.4
104 XCELLERATE I	67	95	3.2	84	78	2.7
104 XCELLERATE II	110	99	3.7	93	87	2.9
107 XCELLERATE I	69	99	2.3	85	82	2.3
107 XCELLERATE II	119	99	2.7	95	92	2.8
110 XCELLERATE I	63	99	2.9	91	82	2.6
110 XCELLERATE II	83	99	3.9	93	92	4.5
115 XCELLERATE I	60	99	1.9	92	91	2.7
115 XCELLERATE II	72	99	2.2	96	94	2.8

* = Purity of $CD3^+$ T cells on day 0 after monocyte-depletion in the XCELLERATE I process or after magnetic concentration in the XCELLERATE II process

Ψ = ratio of $CD4^+$: $CD8^+$ T cells on day 0 after monocyte-depletion in the XCELLERATE I process or after magnetic concentration in the XCELLERATE II process

EXAMPLE 2

EFFICIENCY OF $CD3^+$ T CELL ENRICHMENT, MONOCYTE-DEPLETION AND GRANULOCYTE-DEPLETION

For this study, upon receipt at the Xcyte Therapies Development laboratory, the incoming PBMC apheresis product was washed, split and:

1. For the XCELLERATE I process, a monocyte–depletion step was carried out and the $CD14^+$ monocyte-depleted PBMC were cryopreserved and stored in the vapor phase of a LN_2 freezer (as noted in Example I). On the day of set-up of the XCELLERATE I process, the $CD14^+$ monocyte-depleted PBMC were thawed and the XCELLERATE process initiated with DYNABEADS M-450 CD3/CD28 T as detailed in Example I. The average cellular composition and the average efficiency of $CD3^+$ T cell enrichment, $CD14^+$ monocyte-depletion and granulocyte-depletion for the N = 5 donors in these initial steps is shown in Table 5.1 and the data for each individual donor is shown in Table 5.2.

2. For the XCELLERATE II process, the PBMC apheresis product cells cryopreserved and stored in the vapor phase of a LN_2 freezer. On the day of set-up of the XCELLERATE II process, the cryopreserved PBMC apheresis product cells were thawed and the $CD3^+$ T cells magnetically concentrated and the XCELLERATE II process initiated with DYNABEADS M-450 CD3/CD28 T as detailed in Example I. The average cellular composition and the average efficiency of $CD3^+$ T cell enrichment, $CD14^+$ monocyte-depletion and granulocyte-depletion for the N = 5 donors in these initial steps is shown in Table 5.1 and the data for each individual donor is shown in Table 5.2.

As demonstrated in Tables 5.1 and 5.2, the combination of freeze/thawing of the PBMC apheresis product followed by magnetic concentration of $CD3^+$ T cells direct from the thawed PBMC apheresis product in the XCELLERATE II process configuration results in efficient elimination of $CD14^+$ monocytes and granulocytes (Table 5.1 and Table 5.2). The efficiency of the elimination of the $CD14^+$ monocytes and the granulocytes in the XCELLERATE II process is as good as that of the

XCELLERATE I process with the benefit that it eliminates the need for a separate depletion step using the additional "uncoated" DYNABEADS M-450 T reagent and consistently leads to a higher CD4/CD8 ratio.

Table 5.1: Average (N = 5) efficiency of $CD3^+$ T cell enrichment, $CD14^+$ monocyte-depletion and granulocyte-depletion in the Initial Steps of the XCELLERATE I and the XCELLERATE II Process Configurations

Cell Preparation	Average ± Std. Dev Cellular Composition (%)			
	$CD3^+$	$CD14^+$	Granulocytes	CD4/CD8*
Incoming PBMC apheresis product	49 ± 6	16 ± 3	8 ± 7	2.2 ± 0.3
XCELLERATE I				
Monocyte-depleted PBMC	51 ± 6	5.5 ± 3	5.7 ± 5	2.4 ± 0.6
Freeze/thawed Monocyte-depleted PBMC	64 ± 4	6 ± 3	0.4 ± 0.5	2.4 ± 0.6
XCELLERATE II				
Freeze-thawed PBMC apheresis product	56 ± 5	11 ± 2	0.4 ± 0.5	2.4 ± 0.8
Post- $CD3^+$ magnetic concentration	92 ± 22	2.4 ± 3.7	0 ± 0	2.86 ± 0.86

Cellular compositions were determined by flow cytometry according to standard protocols.

Table 5.2: Comparison of the efficiency of $CD3^+$ T cell enrichment, $CD14^+$ monocyte-depletion and granulocyte-depletion in the initial steps of the XCELLERATE I and the XCELLERATE II process configurations

Experiment Number (Donor)	Cell Preparation	Cellular Composition (%)			
		$CD3^+$	$CD14^+$	Granulocytes	CD4/CD8*
NDa104 (PC071)	Incoming PBMC apheresis product	43%	11%	14%	2.2
	XCELLERATE I				
	Monocyte-depleted PBMC	54%	5%	12.5%	3.2
	Freeze/thawed Monocyte-depleted PBMC	67%	4%	0%	3.2
	XCELLERATE II				
	Freeze-thawed PBMC apheresis product	64%	7%	0%	3.1
	Post- $CD3^+$ magnetic concentration	110%	1%	0%	3.7

Experiment Number (Donor)	Cell Preparation	Cellular Composition (%)			
		$CD3^+$	$CD14^+$	Granulocytes	CD4/CD8*
NDa107 (PC074)	Incoming PBMC apheresis product	51%	16%	1%	2.1
	XCELLERATE I				
	Monocyte-depleted PBMC	64%	5%	1%	2.3
	Freeze/thawed Monocyte-depleted PBMC	69%	3%	0%	2.3
	XCELLERATE II				
	Freeze-thawed PBMC apheresis product	55%	11%	0%	2.0
	Post- $CD3^+$ magnetic concentration	120%	0%	0%	2.7
NDa110 (PC076)	Incoming	44%	18%	15%	2.5
	XCELLERATE I				
	Monocyte-depleted PBMC	63%	3.5%	10%	2.9
	Freeze/thawed Monocyte-depleted PBMC	63%	7%	0%	2.9
	XCELLERATE II				
	Freeze-thawed PBMC apheresis product	55%	13%	0%	3.2
	Post- $CD3^+$ magnetic concentration	83%	1%	0%	3.8
NDa113 (PC060)	Incoming PBMC apheresis product	47%	17%	6%	2.3
	XCELLERATE I				
	Monocyte-depleted PBMC	61%	4%	3%	1.8
	Freeze/thawed Monocyte-depleted PBMC	63%	4%	1%	1.8
	XCELLERATE II				
	Freeze-thawed PBMC apheresis product	51%	13%	1%	1.5
	Post- $CD3^+$ magnetic concentration	76%	1%	0%	1.9
NDa115 (PC073)	Incoming PBMC apheresis product	59%	17%	2%	1.7
	XCELLERATE I				
	Monocyte-depleted PBMC	60%	10%	2%	1.8
	Freeze/thawed Monocyte-depleted PBMC	60%	11%	1%	1.9
	XCELLERATE II				
	Freeze-thawed PBMC apheresis product	53%	12%	1%	2.0
	Post- $CD3^+$ magnetic concentration	72%	9%	0%	2.2

Cellular compositions were determined by flow cytometry according to standard protocols.

In addition to the simplification and streamlining of the process by elimination of the $CD14^+$ monocyte-depletion step and the associated reagents, the magnetic concentration step in the XCELLERATE II™ process also provides a higher

purity of $CD3^+$ T cells and a higher ratio of $CD3^+$ $CD4^+$: $CD3^+$ $CD8^+$ T cells at the initiation of T cell activation (Table 5.1 and Table 5.2).

Yield, Purity, Viability and Composition of Activated $CD3^+$ T cells Pre-harvest on Day 8 of the XCELLERATE I™ process and the XCELLERATE II™ process were also compared.

As shown in Table 5.3, the average yield, purity and viability of the $CD3^+$ T cells prior to harvest on day 8 are typically improved for the XCELLERATE II™ compared to the XCELLERATE I™ process.

Table 5.3: Yield, purity, viability and composition of activated $CD3^+$ T cells pre-harvest on day 8 of the XCELLERATE I process and the XCELLERATE II process

Experiment Number (Donor)	XCELLERATE Process Configuration	Pre-harvest $CD3^+$ T cell Product Properties			
		# $CD3^+$ T cells	Purity $CD3^+$ T cells (%)	Viability (%)	CD4/CD8 Ratio*
NDa104 (PC071)	XCELLERATE I	65×10^9	95	97	1.2
	XCELLERATE II	50×10^9	97	97	1.7
NDa107 (PC074)	XCELLERATE I	57×10^9	98	98	0.8
	XCELLERATE II	52×10^9	98	98	1.5
NDa110 (PC076)	XCELLERATE I	41×10^9	96	96	1.6
	XCELLERATE II	41×10^9	99	99	2.4
NDa113 (PC060)	XCELLERATE I	41×10^9	96	96	1.3
	XCELLERATE II	43×10^9	98	98	2.0
NDa115 (PC073)	XCELLERATE I	31×10^9	96	96	1.3
	XCELLERATE II	48×10^9	97	97	1.4
Average ± Std Dev	XCELLERATE I	47 ± 14	96 ± 2	97 ± 1	1.2 ± 0.3
	XCELLERATE II	45 ± 6	98 ± 1	98 ± 1	1.8 ± 0.4

* = Ratio of $CD3^+$ $CD4^+$: $CD3^+$ $CD8^+$ T cells.

Also, as shown in Table 5.3, the XCELLERATE II™ process maintains a higher ratio of $CD3^+$ $CD4^+$: $CD3^+CD8^+$ T cells throughout the process. This may be

due to preferential concentration of $CD3^+$ $CD4^+$ cells during the magnetic concentration step (Tables 5.1 and 5.2).

"Incoming" refers to fresh, washed incoming apheresis cells. The starting cells listed in Table 5.2 for the XCELLERATE I™ process were apheresed cells that had been washed, monocyte depleted, and/or frozen/thawed. The starting cells listed in Table 5.2 for the XCELLERATE II™ process were apheresis cells that had been washed and frozen/thawed.

* = Ratio of $CD3^+$ $CD4^+$: $CD3^+$ $CD8^+$ T cells

Table 5.3 shows that the XCELLERATE II™ process resulted in a cell product that was more pure (in terms of $\%CD3^+$ cells) than the cell product from the XCELLERATE I™ process. That is, the product cells from the XCELLERATE II™ process had an average (± std dev) $CD3^+$ cell purity of 96% ± 1% while the cells from the XCELLERATE I™ process had an average purity of 93% ± 2%.

Also, as shown in Table 5.3, the XCELLERATE II™ process maintained a higher ratio of CD4/CD8 cells. The incoming cells had an average CD4/CD8 cell ratio of 2.2 and the product cells from the XCELLERATE II™ process had a CD4/CD8 ratio of 1.8, while the product cells from the XCELLERATE I™ process had a CD4/CD8 ratio of 1.2.

The data of Table 5.3 also shows that the XCELLERATE II™ process resulted in product cells with an average viability of 98% while the XCELLERATE I™ process resulted in product cells with an average viability of 97%.

EXAMPLE 3

MONOCYTE DEPLETION

Monocytes ($CD14^+$ phagocytic cells) are removed from T cell preparations via magnetic depletion using a variety of "irrelevant" (*i.e.*, non-antibody coated or non-target antibody coated) Dynal beads. Depletion was performed by pre-incubating either whole blood after separation in ficol or apheresed peripheral blood with Dynal Sheep anti-mouse M-450 beads, or Dynal human serum albumin-coated

beads (M-450), or with Dynal Epoxy (M-450) beads at roughly a 2:1 bead to cell ratio. The cells and beads were incubated for periods of 1-2 hours at 22-37 degrees C, followed by magnetic removal of cells that had attached to beads or that had engulfed beads. The remaining cells were placed into culture alongside un-manipulated cells. Cells were characterized by flow cytometry for cell phenotype before and after depletion.

EXAMPLE 4

FLOW CYTOMETRY SETTINGS

A Becton Dickinson FACSCALIBUR cytometer was used for all the data collected and presented. Any flow cytometer capable of performing 3-color analysis could be used by an experienced operator to acquire identical data. For example, a FACSCAN, Vantage Cell Sorter, or other BD product would work to collect similar data. Also, Coulter products, such as the Coulter Epic Sorter would work as well.

The instrument setting given below can be used as a general guideline for instrument conformation to gather data as was done in these studies. These settings were used for the Examples provided herein; however, modifications to these settings can and should be made by an experienced instrument handler to adjust appropriately for compensation and detector voltages. Also, the use of different detection antibodies with different fluorescent tags requires unique adjustment to any particular instrument to give optimal signal separation (voltage) with minimal "bleeding-over" into other channels (*e.g.*, compensation). A skilled flow operator, well-versed in using compensation controls, isotype controls, and with a general understanding of T cell biology should be able to reproduce any of the data presented below.

Further it should be noted that various settings, particularly voltage settings, may vary, depending upon the efficiency of the instrument laser. For example, older lasers may require more voltage to generate a signal comparable to a newer laser. However, the data obtained, whether with more or less voltage, should reflect similar patterns in biology.

Settings used on the FACSCALIBUR™ (Becton Dickinson):

Detector/Amps:

Parameter	Detector	Voltage	Amp/Gain	Mode
P1	FSC	EOO	1.30	Lin
P2	SSC	370	1.00	Lin
P3	FL1	610	1.00	Log
P4	FL2	550	1.00	Log
P5	FL3	520	1.00	Log

Although the parameter voltages are generally constant, P3, P4, and P5 may be adjusted slightly up or down in order to achieve maximum signal separation, while maintaining a negative control signal value in or near the first decade (0-10) in signal strength in the log mode.

Threshold:
Primary parameter: FSC (forward scatter)
Value: 52

Secondary parameter: none

Compensation:
FL1 – 4.0% FL2
FL2 – 21.4% FL1
FL2 – 2.6% FL3
FL3 – 15.2% FL2

While the settings provided approximate the settings used to collect most of the data presented below, the settings may be altered and roughly equivalent data on stimulated T cells should be generated. The general acceptable ranges for compensation at the voltages listed above are as shown below:

FL1-FL2	0.4-4%
FL2-FL1	18-27%
FL2-FL3	2-8%
FL3-FL2	10-16%

The determination of the particular compensation or voltage values has to be made by an experienced flow cytometer operator with the following goals:

1) Voltage: Maximization of signal separation between positive and negative signals (*e.g.*, surface antigen marker negative vs. low levels surface antigen vs. high levels surface antigen).

2) Compensation: Minimization of interchannel interference (bleed-over) by use of compensation controls.

As voltage settings change, so do compensation settings.

EXAMPLE 5

CELL PROLIFERATION AND VIABILITY ASSAYS

Cell proliferation and viability was measured by standard Trypan Blue staining and cell counting using a hemocytometer. See Figures 5A-5B.

EXAMPLE 6

ACTIVATION MARKER ASSAYS

CD154 is expressed on activated T cells in a temporal manner and has been shown to be a key element in T cells interactions via CD40 on APCs. Blocking the interaction of these two receptors can effectively alter, and even shut-off, an immune response. Aliquots of T cells that were stimulated by concentration with 3x28 paramagnetic beads were removed from cell culture at days 3, 5, and 8 and analyzed for the level of CD154 expression. The level of CD154 expression was compared with T cells that were depleted of monocytes but were not incubated with 3x28 paramagnetic

beads (that is, the T cells were not magnetically concentrated at culture initiation). Significant activation of the T cells stimulated by magnetic concentration with anti-CD3 and anti-CD28 beads was shown by a three-fold increase in the level of CD154 expression on the third day of culture compared with cells that were not similarly stimulated at culture initiation. (See Figures 4 and 7). CD25 levels measured in a similar manner (Figure 6) show a trend toward higher activation.

In general, marker expression was monitored over various times. In this regard cells are labeled with anti-human CD4 (Immunotech, Fullerton, CA), FITC coupled anti-human CD11a (Pharmingen), FITC coupled anti-human CD26 (Pharmingen), FITC coupled anti-human CD49d (Coulter), FITC coupled anti-human CD54 (Pharmingen and Becton Dickinson), FITC coupled anti-human CD95 (Pharmingen), FITC coupled anti-human CD134 (Pharmingen), FITC coupled anti-human CD25 Ab (Becton Dickinson, Fullerton, CA), FITC coupled anti-human CD69 Ab (Becton Dickinson), FITC or PE coupled anti-human CD154 Ab (Becton Dickinson), or FITC or PE coupled IgG1 isotype control Ab. Cells, $2x10^5$ are labeled for 20 minutes at 4°C with 2 µl of each antibody in a final volume of 30 µl, washed and resuspended in 1% parformaldehyde (Sigma, St. Louis, MO).

Comparison of cell surface marker molecule expression levels may be carried out by a variety of methods and thus absolute values may differ. However, when comparing two values the relative fold values may be readily calculated. For example, CD154 expression levels on T cells generated by different "activation" methods can be measured with relative accuracy by flow cytometric means. Using a reagent, such as Becton Dickinson's anti-CD154 –PE conjugate (catalogue # 340477), one can stain T cells in resting or activated states and gauge expression levels for this marker (or others by means well known to experienced flow cytometer operators). Described herein are methods which provide for increased expression of CD154 on T cells, both $CD4^+$ and $CD8^+$. By simultaneously stimulating and concentrating T cells at the initiation of culture, as described herein, expression levels can be driven up beyond values obtained by standard 3x28 activation, on the order of a 20% to over a 100% increase in levels, as measured by mean fluorescent intensity (MFI) using flow

cytometry (BD FACSCalibur and antibody described above). For example, an unstimulated $CD4^+$ T cell would be negative for CD154 and would therefore yield MFI values between 1-10. Upon activation by XCELLERATE I™, at 3 days post-activation, MFI values for CD154 on $CD4^+$ T cells might be in the 20-40 range, while the XCELLERATE II™ process might yield CD154 MFI values of 60-200. While these are not absolute values in terms of the number of CD154 molecules expressed on T cells, there are sufficient to determine relative levels of increased expression. Accordingly, it can be demonstrated that an approximate 1.1 to 20 fold increase in CD154 levels between 1-4 days, post-activation can be demonstrated with the XCELLERATE II™ process as compared to the XCELLERATE I™ process.

EXAMPLE 7

CYTOKINE ASSAYS

Cells are prepared as described above. Supernatants from cells stimulated for various times are subjected to an IL-2, IL-4, INF-gamma or TNF-α ELISA according to the manufacturer's instructions (Biosource International, Sunnyvale, CA).

In an alternative assay, IL-2 is measured by intracellular staining of CD4 T cells using flow cytometry. For intracellular labeling of IL-2 or IFN-γ, cells are first incubated with 1 μml Monensin (Calbiochem) for 4 hours prior to assay. The cells are subsequently stained for surface proteins as described above, fixed and permeabilized using Becton Dickinson intracellular staining-kit, labeled with PE-coupled anti-human IL-2 Ab and FITC coupled anti-human IFN-γ or the corresponding control Abs as described by the manufacturer. Data acquisition and flow cytometric analysis is performed on a Becton Dickinson FACSCalibur flow cytometer using Cellquest software following the manufacturer's protocol (Becton Dickinson).

IFN-gamma concentrations were about 2, 3, 4, and in some cases 5 fold higher at day 3 when using the XCELLERATE II™ methodology as opposed to XCELLERATE I™ (data not shown). Further, TNF-alpha levels were also markedly

higher (between 1.5 to 3 fold higher) up to day 5 following stimulation (data not shown) as compared with XCELLERATE I™.

EXAMPLE 8

PHENOTYPICAL CELL ANALYSIS AFTER RESTIMULATION

For restimulation analysis about $5x10^6$ cells are taken from the culture at the day of termination. In several examples, the date of termination is day 8 of culture. The cells are placed into 5 mL of X-vivo 15 media with serum and with or without IL-2 as indicated above, in one well of a six well plate. About $5x10^6$ Dynabeads M-450 CD3/CD28 T beads to the well containing the cells and the cells and beads are placed in a 37°C, 5% CO_2 incubator. After two days, the samples are removed and tested for viability and analyzed by FACS to determine cell size, and cell marker and/or cytokine expression levels, such as CD25 expression levels, CD154 expression levels. Table 6 demonstrates these results below for five patient samples subject to the XCELLERATE I™ and the XCELLERATE II™ process.

Table 6: Results of the Re-stimulation Assay for XCELLERATED T cells Produced Using the XCELLERATE I™ and the XCELLERATE II™ Processes

Experiment Number (Donor)	Process Configuration	Cell Size (FSC)		CD25 (MFI)		CD154 (MFI)	
		T = 0	T = 48 hr	T = 0	T = 48 hr	T = 0	T = 48 hr
NDa104 (PC071)	XCELLERATE I	393	607	104	478	6	37
	XCELLERATE II	404	659	115	544	12	70
NDa107 (PC074)	XCELLERATE I	386	596	59	585	6	121
	XCELLERATE II	380	607	62	721	10	109
NDa110 (PC076)	XCELLERATE I	425	501	111	600	10	39
	XCELLERATE II	390	445	97	434	15	36
NDa113 (PC060)	XCELLERATE I	399	630	66	659	8	32
	XCELLERATE II	411	633	113	816	12	145

Experiment Number (Donor)	Process Configuration	Cell Size (FSC)		CD25 (MFI)		CD154 (MFI)	
		T = 0	T = 48 hr	T = 0	T = 48 hr	T = 0	T = 48 hr
NDa115 (PC073)	XCELLERATE I	433	514	105	247	13	10
	XCELLERATE II	408	569	81	369	20	36
Average ± Std Dev (n = 5)	XCELLERATE I	407 ± 21	570 ± 58	89 ± 24	514 ± 163	9 ± 3	48 ± 43
	XCELLERATE II	399 ± 13	583 ± 84	94 ± 22	577 ± 189	14 ± 4	79 ± 48

EXAMPLE 9

ALTERNATIVE CELL COLLECTION AND CULTURE PROTOCOLS

XCELLERATE™

Cells isolated from human blood are grown in X-vivo media (Biowhittaker Inc., Walkersville, MD) and depending on use supplemented with or without 20 U/ml IL-2 (Boehringer Mannheim, Indianapolis, IN) and supplemented with 5% human serum (Biowhittaker), 2 mM Glutamine (Life Technologies, Rockville, MD) and 20 mM HEPES (Life Technology). Jurkat E6-1 cells (ATCC, Manassas, VA) are grown in RPMI 1640 (Life Technologies) supplemented with 10% FBS (Biowhittaker), 2 mM glutamine (Life Technologies), 2 mM Penicillin (Life Technologies), and 2 mM Streptomycin (Life Technologies).

Buffy coats from healthy human volunteer donors are obtained (American Red Cross, Portland, OR). Peripheral blood mononuclear cells (PBMC) are obtained using Lymphocyte Separation Media (ICN Pharmaceuticals, Costa Mesa, CA) according to the manufacturers' instructions.

Peripheral blood lymphocytes (PBL) are obtained from the PBMC fraction by incubation in culture flask (Costar, Pittsburgh, PA) with uncoated Dynabeads (Dynal, Oslo, Norway), 10^8 cells/ml, 2 beads/cell, 2h at 37°C. Monocytes and macrophages can be removed by adherence to the culture flask. Alternatively, they

can be removed by phagocytosing the paramagnetic beads and then depleting these cells by magnetic cell separation according to the manufacture's instruction (Dynal). $CD4^+$ cells are purified from the PBL fraction by incubation with 10 μg/ml of monoclonal antibodies against CD8 (clone G10-1), CD20 (clone IF5), CD14 (clone F13) and CD16 (Coulter), 10^8 cells/ml, 20 min at 4°C. After washing, cells are treated with sheep anti-mouse Ig-coupled Dynabeads (10^6 cells/ml, 6 beads/cell, 20 min at 4°C) and then depleted twice via magnetic cell separation. The purity of $CD4^+$ cells are routinely 91-95% as measured by Flow cytometry.

Dendritic cells are generated by first adhering PBMC to a culture flask (Costar), 10^8 cells/ml, 2h at 37°C (without Dynabeads). After extensive washing, adherent cells are cultured for 7 days in media containing 500 U/ml GM-CSF (Boehringer Mannheim) and 12.5 U/ml IL-4 (Boehringer Mannheim). The resulting cell population is weakly adherent and expresses surface markers characteristic of dendritic cells (e.g., expresses HLA-DR, CD86, CD83, CD11c and lacks expression of CD4). (All antibodies obtained from Becton Dickinson, San Jose, CA).

Other techniques can utilize human peripheral blood lymphocytes containing T cells that are incubated in tissue culture plates and/or tissue culture flasks (Baxter bags), or other common culture vessels in media, which could be composed of RPMI, X-Vivo 15, or some other T cell culture media. Although not required for the activation and growth of T cells, glutamine and HEPES are added to the culture media. Fetal bovine serum (10% final), human A/B serum (5%), or autologous human serum (5%) is added to culture media. The percentage of serum may vary without greatly affecting T cell biology or culture outcome. In some instances, recombinant human IL-2 is added to cultures. In some instances, phagocytic $CD14^+$ cells and other phagocytic cells are remove by magnetic depletion as described, *infra*. Beads having co-immobilized upon their surface anti-CD3 and anti-CD28 (3x28 beads) are added at a 3:1 bead:cell ratio. In some instances, 3x28 beads are added at a 1:1 bead:cell ratio. In other instances, the 3x28 beads are added sequentially over the first 5 days of culture with final ratios of 1:1 at day 1, 1:5 at days 3 and 5. Cultures are maintained at 37 degrees C at 5-7% CO_2. Cells are removed at several timepoints over a 14 day period

to determine cell density (cell number), cell size, and cell surface phenotype as measured via flow cytometric analysis of a variety of surface antigens. Supernatants are also collected from cultures to determine cytokine secretion profiles, including, but not limited to: IL-2, IL-4, IFN-γ , TNF-α. As activated cells grow and divide, cultures are maintained at 0.2-2x10^6 $CD3^+$ T cells/ml. When T cell density exceeds roughly 1.5x10^6/ml, cultures are split and fed with fresh media so as to give a cell density in the 0.2-1.4x10^6/ml range. At roughly 2 hours to about 14 days following initial stimulation, when activated T cells are shown to be entering a more quiescent phase (*e.g.*, CD25 levels diminishing, cell size as determined by forward scatter is diminishing, rate of cell division may be reduced), cells are either infused into the subject or re-stimulated with one of the following stimuli:

1) No stimulus
2) Phytohemagglutinin (PHA) 2 μg/ml
3) (3x28 beads) at a 1:1 bead/cell ratio

Cells are again analyzed over time for cell phenotype and activation/functional state. Supernatants are again collected for secreted cytokine analysis.

Cells were stimulated by three different methodologies 1) Dynabeads (M-450) covalently coupled to anti-CD3 (OKT-3) and anti-CD28 (9.3) antibodies (3x28 beads) according to the manufacturer's instructions (Dynal), 3 beads/cell, 2) Ionomycin (Calbiochem, La Jolla, CA) (100 ng/ml) and phorbol 12-myristate-13-acetate (PMA) (Calbiochem) (10 ng/ml), 3) allogeneic dendritic cells (25,000 dendritic cells/200,000 CD4 cells). All cells are stimulated at a concentration of 10^6 cell/ml. Proliferation assays are conducted in quadruplicate in 96 well flat-bottom plates. Cells are stimulated at 10^6 cells/ml in a final volume of 200 μl. Proliferation is measured by MTT assay (MTT assay kit, Chemicon International Inc., Temecula, CA) at day 3 (stimulation method 1 and 2) or at day 6 (stimulation method 3), and results are presented as mean value of quadruplicates. PBL cultures or purified $CD4^+$ cell cultures are stimulated with 3x28 beads, ionomycin/PMA, or allogeneic dendritic cells.

As demonstrated by Figures 8A-8B, cell numbers (Coulter counter) increase dramatically following stimulation with PHA, 3x28 beads (anti-CD3 and anti-CD28 co-immobilized on beads) attached to the beads via sheep anti-mouse (SAM), 3x28 beads with the antibodies covalently attached to the beads, or antibodies singly or dually immobilized on a plate. Figure 9 also demonstrates increases in cell numbers following stimulation with covalently immobilized anti-CD3 and anti-CD28 on beads +/- monocyte depletion and +/- 20 units of IL-2.

EXAMPLE 10

MONOCYTE DEPLETION VIA MAGNETIC DEPLETION

Monocytes ($CD14^+$ phagocytic cells) are removed from T cell preparations via magnetic depletion using a variety of "irrelevant" (i.e., non-antibody coated or non-target antibody coated) Dynal beads. Depletion was performed by pre-incubating ficolled whole blood, or apheresed peripheral blood with roughly 2:1 bead to cell ratio of Dynal Sheep anti-mouse M-450 beads, or Dynal human serum albumin-coated beads (M-450), or with Dynal Epoxy (M-450) beads for periods of 1-2 hours at 22-37 degrees C, followed by magnetic removal of cells which had attached to beads or engulfed beads. The remaining cells were placed into culture alongside un-manipulated cells. Cells were characterized by flow cytometry for cell phenotype before and after depletion. Figure 9 demonstrates increased proliferation in the absence of monocytes.

EXAMPLE 11

PRE-ACTIVATION AND POST-ACTIVATION KINETIC TIMECOURSE STUDIES

A series of experiments were performed in which human T cells, isolated either from whole blood or from apheresed peripheral blood, were cultured under a variety of conditions. Those conditions include:

1) No stimulation
2) Stimulation with phytohemagglutinin (PHA) at 2 μg/ml.

3) Stimulation with 3x28 Dynabeads (beads having anti-CD3 and anti-C28 beads conjugated thereto) at 3:1 or 1:1 bead-to-T cell ratio.

4) Stimulation or culture in the presence or absence of exogenously added recombinant human IL-2 at 10 U/ml (5 ng/ml).

5) Culture in the presence of monocytes ($CD14^+$ phagocytic cells) or cultured following removal of aforementioned cells via magnetic depletion using a variety of "irrelevant" Dynabeads. Depletion was performed as illustrated in Example 2.

The following cell surface markers were analyzed by flow cytometry to determine cell phenotype and activation state: CD2, CD3, CD4, CD8, CD14, CD19, CD20, CD25, CD45RA, CD45RO, CD54, CD62L, CDw137 (41BB), CD154. Cell size is also examined, as determined by forward scatter profiles via flow cytometry.

Markers, such as CD2, CD3, CD4, CD8, CD14, CD19, CD20, CD45RA, and CD45RO are used to determine T, B, and monocyte lineages and subpopulations, while forward scatter, CD25, CD62L, CD54, CD137, CD154 are used to determine activation state and functional properties of cells.

Human peripheral blood lymphocytes containing T cells were prepared as described in Example IX. Cells are analyzed over time for cell phenotype and activation/functional state. Supernatants are collected for secreted cytokine analysis. Figures 8 and 9 demonstrates general growth characteristics of human T cells following activation with 3x28 beads +/- recombinant human IL-2 at 10u/ml and +/- monocyte depletion . All cells were cultured in Baxter Lifecell Flasks (300ml). The one plot labeled "Scale up" refers to a 300ml flask culture (No IL-2/Monocyte depleted) that was expanded up to a Baxter Lifecell 3 liter flask. The graph demonstrates an approximate 2-4 log expansion of human T cells under the various conditions.

Figure 10 shows the kinetic analysis of cell size as determined by forward scatter flow cytometry profiles over time. T cell are seen to increase in size shortly after activation and subsequently decrease in size so that by day 14 they demonstrate smaller forward scatter profiles, indicating a more quiescent state.

Figure 11 shows IL-2 receptor (CD25) expression over time following 3x28 bead stimulation. Both $CD4^+$ and $CD8^+$ T cells show an early increase in receptor

level. By day 14, CD25 expression levels are greatly reduced on a majority of T cells, indicating a more quiescent state.

When 3x28-stimulated T cells became more quiescent (low CD25, low forward scatter), they were re-stimulated as shown below:

1) No stimulation
2) PHA 2ug/ml
3) 3x28 (Xcellerate) bead stimulation at 1 bead/$CD3^+$ T cell

A kinetic analysis of cell size (forward scatter), surface phenotype, activation marker expression, and cytokine secretion was then performed. Figure 12 shows forward scatter (cell size) kinetics following primary and secondary stimulation. Figure 13 shows CD25 (IL-2-Receptor) expression kinetics following primary and secondary stimulation. Figure 16 shows CD54 (I-CAM) expression following secondary stimulation, on $CD4^+$ T cells (A) and on $CD8^+$ T cells (B), where the primary stimulation was either PHA or 3x28 beads, and re-stimulation was either: none, PHA, or 3x28 beads. Markers delineating between CD4 and CD8 positive cells were also used to determine their relative proportion during 3x28 antibody bead activation (Figures 19 and 22).

EXAMPLE 12

ANALYSIS OF CYTOKINE EXPRESSION PATTERNS OF CO-STIMULATED T CELLS

The role of a variety of cytokines, including IL-2, IFN-γ, TNF-α, and IL-4 have been extensively studied as they relate to T cell maintenance, expansion, and differentiation. Notably, IL-2 has been shown to be supportive of T cell maintenance and expansion. IFN-γ has been implicated in driving T cells to differentiate into T_{H1}-type immune responder, while IL-4 has been implicated for driving T cells to T_{H2}-type responses. Cytokine release levels in primary human T cells activated by either PHA or

3x28 beads were analyzed by stimulating T cells as in Example IX, including kinetic studies of responses to primary stimulation and responses to a secondary stimulus. The data are shown in Figures 18A-C and Figures 23-24 demonstrate a unique feature of 3x28 bead stimulation. Between day 2 and day 4 following initial stimulation (day one was not assessed), extremely high levels of both IL-2 and IFN-γ were observed. A nearly 5-fold increase in absolute secreted IL-2 levels was seen for 3x28 bead-stimulated T cells as compared to levels observed for cells stimulated with PHA. An approximate 7-fold increase in IFNγ levels was also observed in 3x28 stimulated T cells as compared to their PHA counterparts. In the case of IL-4, the increase was not as dramatic for primary stimulation. Interestingly, and of possibly great significance, is that after cells became quiescent (no longer dividing or secreting the three cytokines mentioned above) following primary stimulation, they were re-stimulated with either 3x28 beads, PHA, or left un-stimulated. T cells which had received an initial activation/expansion signal through 3x28 beads secreted even higher levels of IFN-γ than observed following primary stimulation. In contrast, cells that were initially stimulated with PHA secreted IFN-γ levels much lower than seen for their 3x28 counterparts. Similar difference were also observed for IL-4 levels.

These data suggest that cells obtained following activation/expansion mediated through 3x28 beads are functionally different than those obtained from other means of expansion, such as PHA. The resultant cells appear to have an altered cytokine secretion response, one that promotes very high levels of both T_{H1} and T_{H2} cytokines, with a possible favoring of the T_{H1}-type profile (IFN-γ). Secretion of such high levels of these cytokines in culture can have many effects, including: driving T cells into a T_{H1} differentiation pathway, which is one that favors anti-tumor and anti-viral responses; and also by altering the basic functionality of resultant T cells (such as lowering threshold of activation and inhibiting programmed cell death pathways).

EXAMPLE 13

ANALYSIS OF CD54 EXPRESSION OF CO-STIMULATED T CELLS

Figure 16 shows CD54 (I-CAM) expression following secondary stimulation, on $CD4^+$ T cells (A) and on $CD8^+$ T cells (B), where the primary stimulation was either PHA or 3x28 beads, and re-stimulation was either: none, PHA, or 3x28 beads.

EXAMPLE 14

SHORT TERM ACTIVATION MARKER ASSAYS

Marker expression was monitored over various times following stimulation of T cells as set forth in Example IX. In this regard cells are labeled with anti-human CD4 (Immunotech, Fullerton, CA), FITC-coupled anti-human CD11a (Pharmingen), FITC-coupled anti-human CD26 (Pharmingen), FITC-coupled anti-human CD49d (Coulter), FITC-coupled anti-human CD54 (Pharmingen and Becton Dickinson), FITC-coupled anti-human CD95 (Pharmingen), FITC-coupled anti-human CD134 (Pharmingen), FITC-coupled anti-human CD25 Ab (Becton Dickinson, Fullerton, CA), FITC-coupled anti-human CD69 Ab (Becton Dickinson), FITC- or PE-coupled anti-human CD154 Ab (Becton Dickinson), or FITC-or PE-coupled IgG1 isotype control Ab. Cells, $2x10^5$ are labeled for 20 minutes at 4°C with 2 µl of each antibody in a final volume of 30 µl, washed and resuspended in 1% paraformaldehyde (Sigma, St. Louis, MO). See Figures 21-22, and 26A-26L, as is demonstrated by these figures there appear significant differences over activation time as well as between $CD4^+$ and $CD8^+$ cells.

EXAMPLE 15

T CELL EXPANSION USING VARYING CD3:CD28 RATIOS

T cell expansion was evaluated using varying concentrations of CD3:CD28 ratios on the 3x28 DYNABEADS® M-450. In the experiments described herein, the process referred to as XCELLERATE II™ was used, as described in Example I. As shown in Figure 27, surprisingly, about a 68-fold expansion after 8 days of culture was observed with a CD3:CD28 ratio of 1:10 on the beads. A 35-fold expansion of T cells was seen after 8 days of culture with a CD3:CD28 ratio of 1:3 on the beads. At a 1:1 ratio, about a 24-fold expansion was seen.

EXAMPLE 16

XCELLERATED T CELL THERAPY TO ACCELERATE HEMATOLOGIC RECOVERY

This example describes early results from a Phase I/II Clinical Trial in which patients are receiving high dose myeloablative chemotherapy followed by an autologous peripheral blood stem cell transplant further followed by infusion of Xcellerated T cells.

A Phase I/II Clinical Trial of patients with multiple myeloma was conducted in which patients received high dose myeloablative chemotherapy consisting of 200 milligrams per meter squared followed by an autologous peripheral blood stem cell transplant. After this regimen, patients are typically neutropenic (*i.e.*, have a neutrophil count below 500 per ul for about 8 days). In this clinical trial, patients were administered one infusion of Xcellerated T Cells on day 3 after the transplant. Of the first 20 patients that received the transplant and the infusion of Xcellerated T cells, the overall patient average is about 3 days of neutropenia. Several patients appear never to have had any neutropenic period at all, which is highly unusual if not unheard of (for example, an analysis of historical data in a series of 142 patients receiving the same treatment/transplant, the least number of days of neutropenia in those 142 patients was

four days). These preliminary data indicate that the Xcellerated T Cells speed up not only neutrophil recovery but also recovery of platelets and maybe even red blood cells. Further, the results indicated that treatment with Xcellerated T cells led to rapid lymphocyte recovery including rapid $CD4^+$ and $CD8^+$ T cell recovery. Additionally, as described further in U.S. Application No. 10/360,507 filed February 7, 2003, the results indicated that the Xcellerate process reduces TCR Vβ skewing and restores T cell repertoire in multiple myeloma patients.

EXAMPLE 17

XCELLERATED T CELL THERAPY TO ACCELERATE HEMATOLOGIC RECOVERY IN CHRONIC LYMPHOCYTIC LEUKEMIA

This example describes early results from a Phase I/II Clinical Trial in which CLL patients received infusions of Xcellerated T cells.

Patients enrolled in the study had a high risk or symptomatic/progressive intermediate risk disease & Eastern Cooperative Oncology Group (ECOG) performance status (PS) 0-2. PBMC were collected by leukapheresis for the Xcellerate™ Process as described herein, and patients subsequently receive a single infusion of Xcellerated™ T Cells. Cohorts of 3 patients each were treated with increasing cell doses: 10×10^9, 30×10^9, and $60\text{-}100 \times 10^9$.

Seventeen of approximately 18 planned patients have been treated to date. Data were available for 14 patients, with last f/u visit at a median of 12 weeks (range 8-12). Baseline characteristics [median (range)] were: age 57 (39-68), years from diagnosis 3.9 (1.8-8.7), WBC x $10^3/mm^3$ 56 (6-274). Prior treatments included chemotherapy ± monoclonal antibody treatment (8), investigational vaccine treatment (3), and no prior therapy (3). After the first cohort, a new bioreactor process was instituted in which T cells expanded 98 +/- 31 fold, were 98.2 +/- 1.3% T cells, with a CD4:CD8 ratio of 15.0 + 28.4 (n= 9; mean +/- SD). There have been no serious adverse

events related to treatment to date. Following treatment, T cell counts increased. Increases in neutrophil, platelet, and hemoglobin were also observed (Figures 30A, 30B, and 30C)). Further, increases in NK cell counts were seen. In particular, in at least one case, NK cell counts were below 400/mm^3 at the time of infusion and increased to over 600/mm^3 over the first 90 days following treatment. A significant reduction in lymph node area was observed in 12 of 14 evaluable patients. Median (range) spleen measurement in cm below left costal margin decreased from 3 (0-10) prior to treatment to <1 (0-4) at the time of last follow-up. Decreases in peripheral leukemic cell counts have not been observed to date.

In conclusion, these results indicate that Xcellerated^{TM} T Cells can be manufactured reproducibly and are well tolerated in doses of up to 100 x 10^9 cells. Treatment leads to significant increases in T cell counts, increases in neutrophil, platelet, hemoglobin, and NK cell counts, and significant decreases in lymphadenopathy & splenomegaly.

EXAMPLE 18

XCELLERATED T CELL THERAPY TO ACCELERATE HEMATOLOGIC RECOVERY IN HORMONE-REFRACTORY PROSTATE CANCER

This example describes early results from a Phase I/II Clinical Trial in which hormone-refractory prostate cancer patients received infusions of Xcellerated T cells.

Patients with androgen independent prostate cancer and no history of prior chemotherapy were enrolled. Patients received one infusion of 75-100 x 10^9 CD3/CD28 activated T cells (Xcellerated T Cells). The objectives of the study were to assess the safety of the therapy, changes in serum prostate specific antigen (PSA), and changes in markers of bone resorption.Twenty patients underwent leukapheresis, and 19 were treated (1 patient progressed prior to treatment). Baseline characteristics for the

treated patients [median(range)] were: age 71.1 (55.1-84.4), PSA 28.2 ng/mL (6.2-348.0), and Gleason score 7 (4-9). Ten patients had documented bone metastases. Xcellerated T Cells were successfully manufactured in all patients. The final products were 99.0% (93.0-99.0%) T cells [median (range)] with CD4:CD8 ratio of 3.5 (0.9-15.7). The number of viable cells infused was 96.4 x 10^9 (49.6 x 10^9 – 96.4 x 10^9). Toxicities possibly, probably or definitely related to the therapy of Grade 1, 2 or 3 severity were seen in 13, 3 and 1 patients respectively. The most common toxicities were rigors, pyrexia and nausea. The lymphocyte count per mm^3 increased from 1,218 $\pm$ 156 at Day 0, to 3,455 $\pm$ 355 at Day 7 and 2,756 $\pm$ 393 at Month 4 (mean + SEM). Two patients had PSA declines of >50%, with PSA nadirs occurring approximately 4 and 13 months following treatment. Serum markers of bone resorption (NTX, BAP, ICTP) measured in 5 patients with positive bone scans were not statistically different at Month 3 compared with baseline.The results indicated that Xcellerated T Cells can be delivered on an outpatient basis with few side effects, and resulted in marked and sustained increases in lymphocyte counts as well as increases in neutrophil counts. Significant PSA declines were also observed in some patients.

All of the above U.S. patents, U.S. patent application publications, U.S. patent applications, foreign patents, foreign patent applications and non-patent publications referred to in this specification and/or listed in the Application Data Sheet, including but not limited to U.S. Patent Application No. 09/960,264, filed September 20, 2001; which is a continuation-in-part of U.S. Application No. 09/794,230, filed February 26, 2001; which claims the benefit of Provisional Application Nos. 60/184,788, filed February 24, 2000, and 60/249,902, filed November 17, 2000, are incorporated herein by reference, in their entirety.

From the foregoing it will be appreciated that, although specific embodiments of the invention have been described herein for purposes of illustration, various modifications may be made without deviating from the spirit and scope of the invention. Accordingly, the invention is not limited except as by the appended claims. All of references, patents, patent applications, etc. cited above, are incorporated herein

in their entirety. Further, all numerical ranges recited herein explicitly include all integer values within the range.

CLAIMS

What is claimed is:

1. A method for accelerating hematologic recovery in a patient exhibiting reduced hematologic function, comprising:

(i) contacting a population of cells from the patient wherein at least a portion thereof comprises T cells with a surface, wherein said surface has attached thereto a first agent that ligates a first cell surface moiety of a T cell, and the same or a second surface has attached thereto a second agent that ligates a second moiety of said T cell, wherein said ligation by the first and second agent induces proliferation of said T cell

(ii) administering to the patient the population of T cells of (i);

thereby accelerating hematologic recovery in the patient.

2. The method of claim 1 wherein the first agent comprises an anti-CD3 antibody or an antigen binding fragment thereof and said second agent comprises a ligand which binds an accessory molecule on the surface of said T cells.

3. The method of claim 2 wherein said accessory molecule is CD28.

4. The method of claim 1 wherein the first agent comprises an anti-CD3 antibody or an antigen binding fragment thereof and said second agent comprises an anti-CD28 antibody or an antigen binding fragment thereof.

5. The method of claim 1 wherein said first and second agents are attached to said surface or said second surface by covalent attachment.

6. The method of claim 1 wherein said first and second agents are attached to said surface or said second surface by direct attachment.

7. The method of claim 1 wherein said first and second agents are attached to said surface or said second surface by indirect attachment.

8. The method of claim 1 wherein the patient is afflicted with a cancer.

9. The method of claim 8 wherein the cancer is selected from the group consisting of multiple myeloma, prostate cancer, and chronic lymphocytic leukemia.

10. The method of claim 1 wherein the hematologic recovery comprises one or more of an increase in neutrophil counts, an increase in platelet counts, an increase in hemoglobin levels, and an increase in NK cell counts.

11. A method for accelerating neutrophil recovery in a patient, comprising:

(i) contacting a population of cells from the patient wherein at least a portion thereof comprises T cells with a surface, wherein said surface has attached thereto a first agent that ligates a first cell surface moiety of a T cell, and the same or a second surface has attached thereto a second agent that ligates a second moiety of said T cell, wherein said ligation by the first and second agent induces proliferation of said T cell

(ii) administering to the patient the population of T cells of (i);

thereby accelerating neutrophil cell recovery in the patient.

12. The method of claim 11 wherein the first agent comprises an anti-CD3 antibody or an antigen binding fragment thereof and said second agent comprises a ligand which binds an accessory molecule on the surface of said T cells.

12. The method of claim 11 wherein said accessory molecule is CD28.

14. The method of claim 11 wherein the first agent comprises an anti-CD3 antibody or an antigen binding fragment thereof and said second agent comprises an anti-CD28 antibody or an antigen binding fragment thereof.

15. The method of claim 11 wherein the patient is afflicted with cancer.

16. The method of claim 15 wherein the cancer is selected from the group consisting of multiple myeloma, prostate cancer, and chronic lymphocytic leukemia.

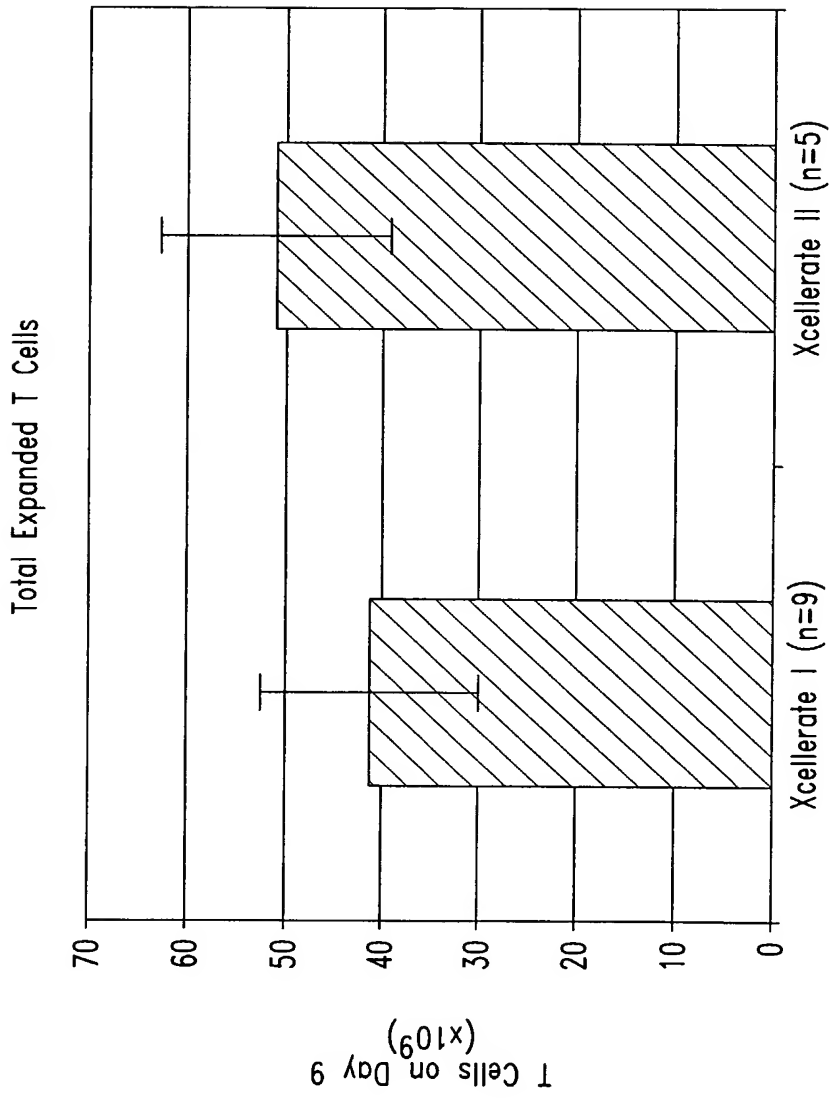

Total Expanded T Cells
T Cells on Day 9
(x10⁹)
70
60
50
40
30
20
10
0
Xcellerate I (n=9)
Xcellerate II (n=5)

Fig. 1

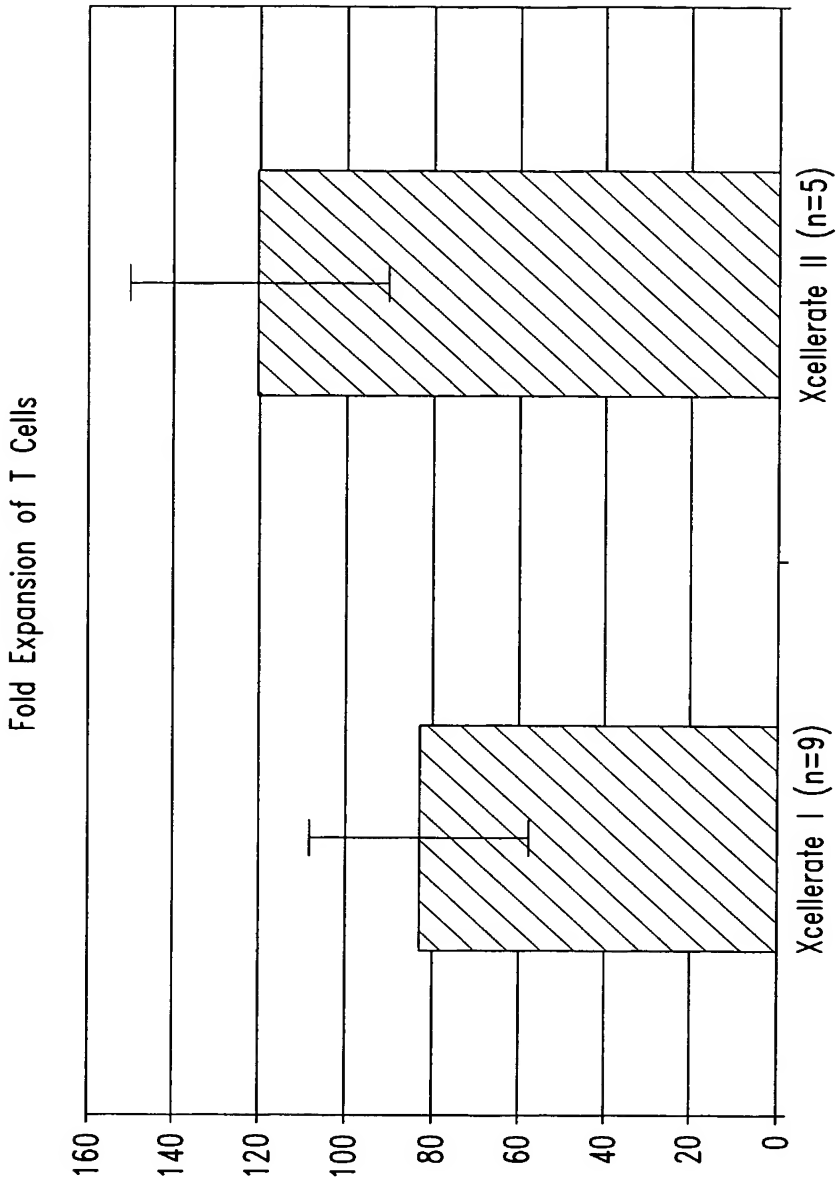

Fold Expansion of T Cells
160
140
120
100
80
60
40
20
0
Xcellerate I (n=9)
Xcellerate II (n=5)

Fig. 2

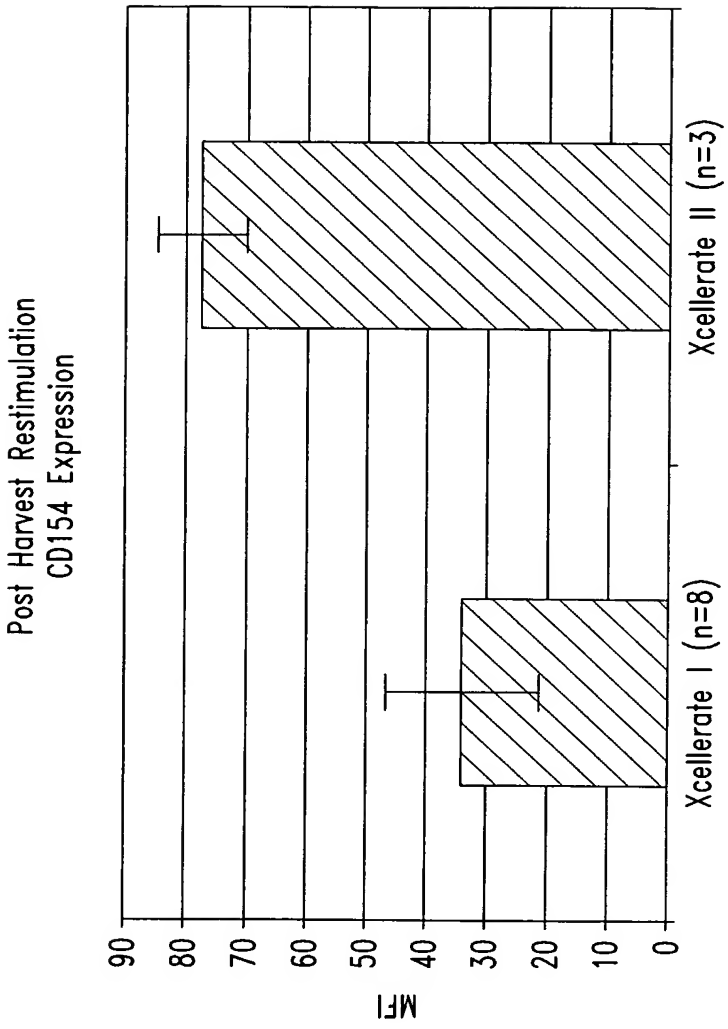

Post Harvest Restimulation
CD154 Expression
MFI
90
80
70
60
50
40
30
20
10
0
Xcellerate I (n=8)
Xcellerate II (n=3)

Fig. 3

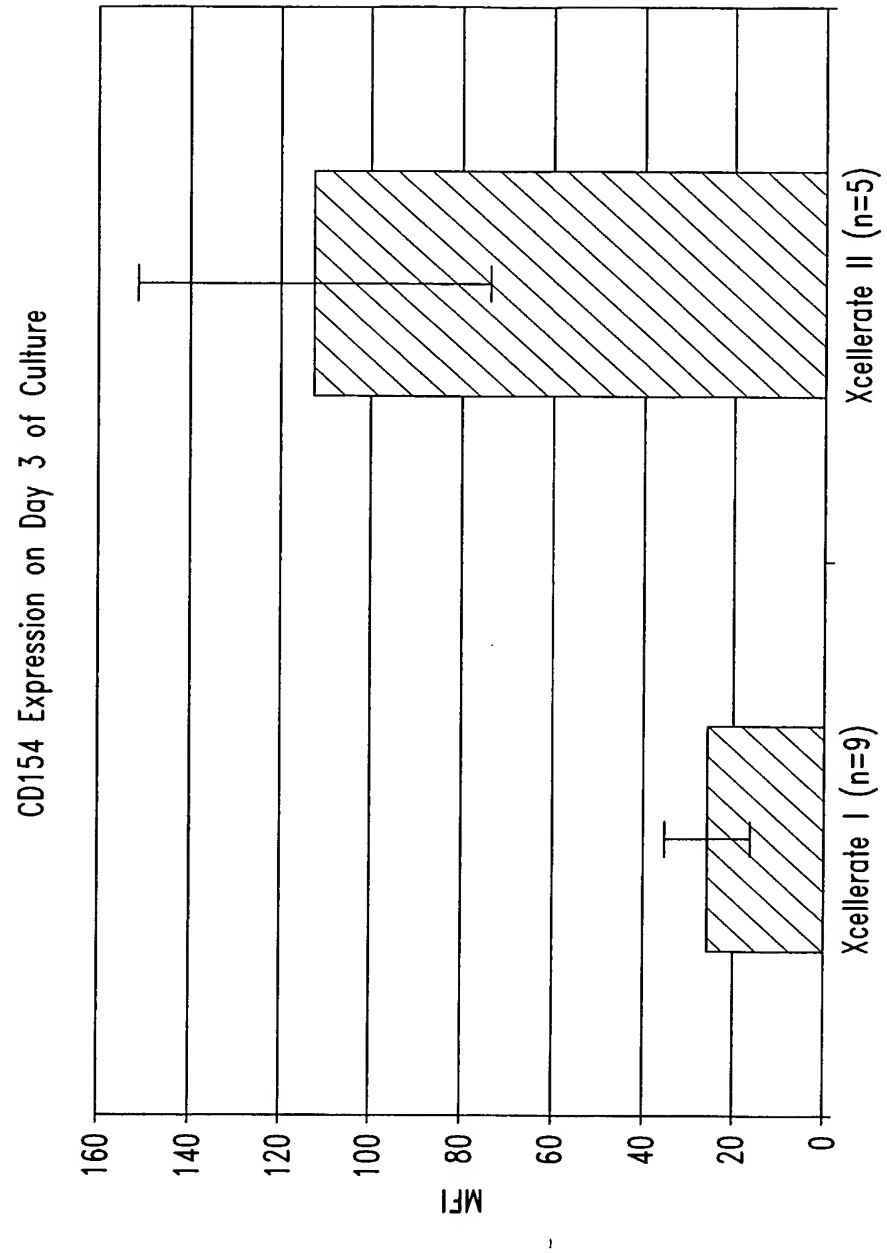

CD154 Expression on Day 3 of Culture
MFI
160
140
120
100
80
60
40
20
0
Xcellerate II (n=5)
Xcellerate I (n=9)

Fig. 4

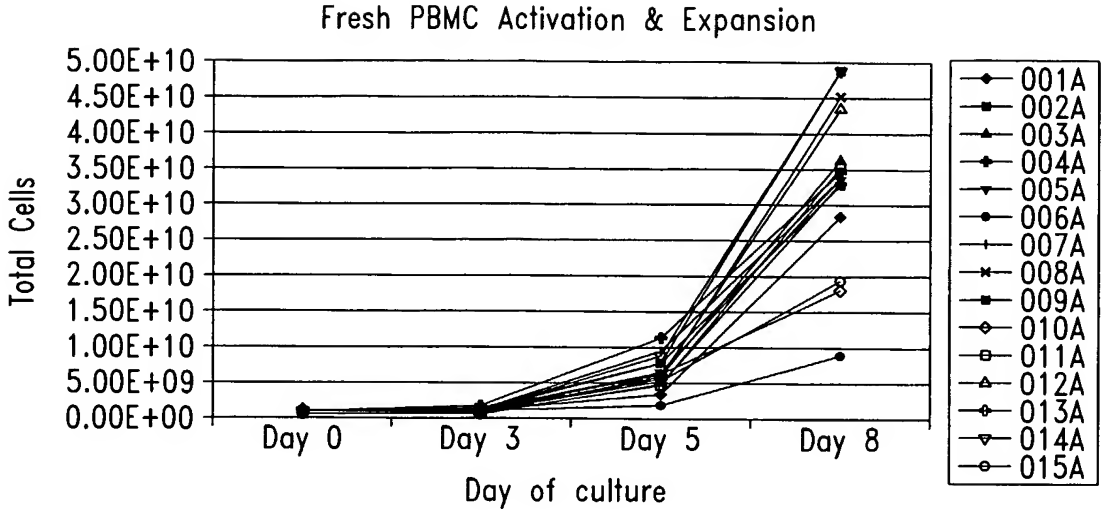

Fresh PBMC Activation & Expansion
Total Cells
5.00E+10
4.50E+10
4.00E+10
3.50E+10
3.00E+10
2.50E+10
2.00E+10
1.50E+10
1.00E+10
5.00E+09
0.00E+00
Day 0
Day 3
Day 5
Day 8
Day of culture
001A
002A
003A
004A
005A
006A
007A
008A
009A
010A
011A
012A
013A
014A
015A

Fig. 5A

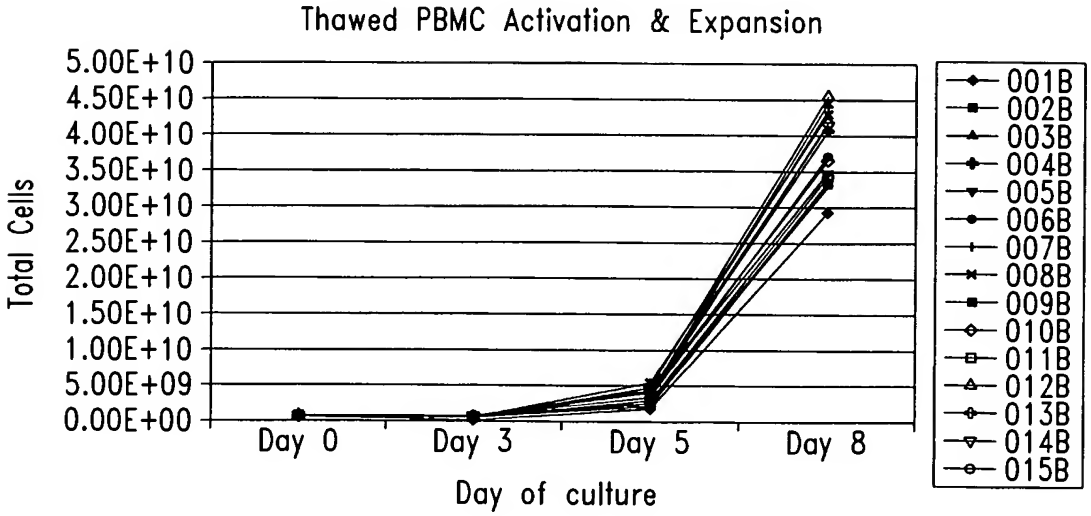

Thawed PBMC Activation & Expansion
Total Cells
5.00E+10
4.50E+10
4.00E+10
3.50E+10
3.00E+10
2.50E+10
2.00E+10
1.50E+10
1.00E+10
5.00E+09
0.00E+00
Day 0
Day 3
Day 5
Day 8
Day of culture
001B
002B
003B
004B
005B
006B
007B
008B
009B
010B
011B
012B
013B
014B
015B

Fig. 5B

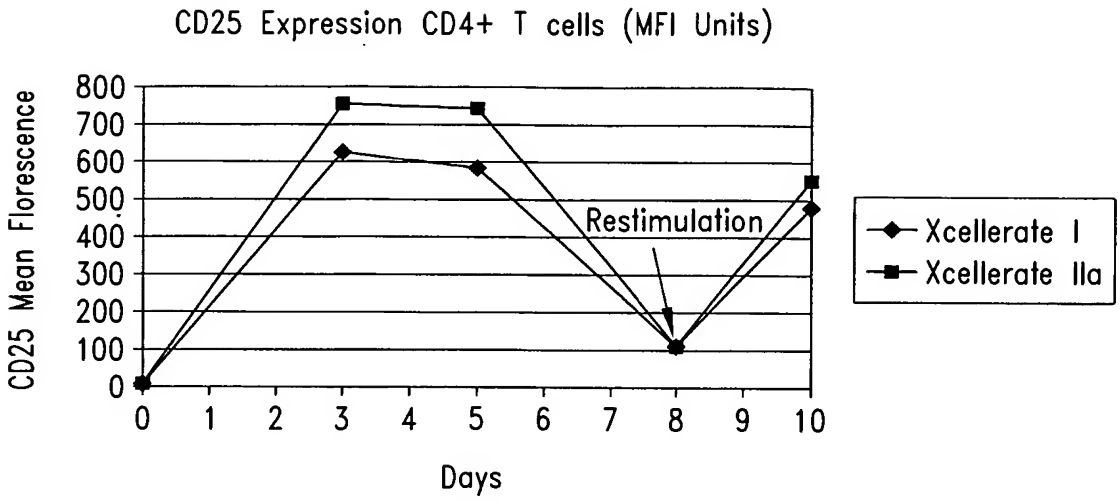

CD25 Expression CD4+ T cells (MFI Units)
CD25 Mean Florescence
800
700
600
500
400
300
200
100
0
0 1 2 3 4 5 6 7 8 9 10
Days
Restimulation
Xcellerate I
Xcellerate IIa

Fig. 6A

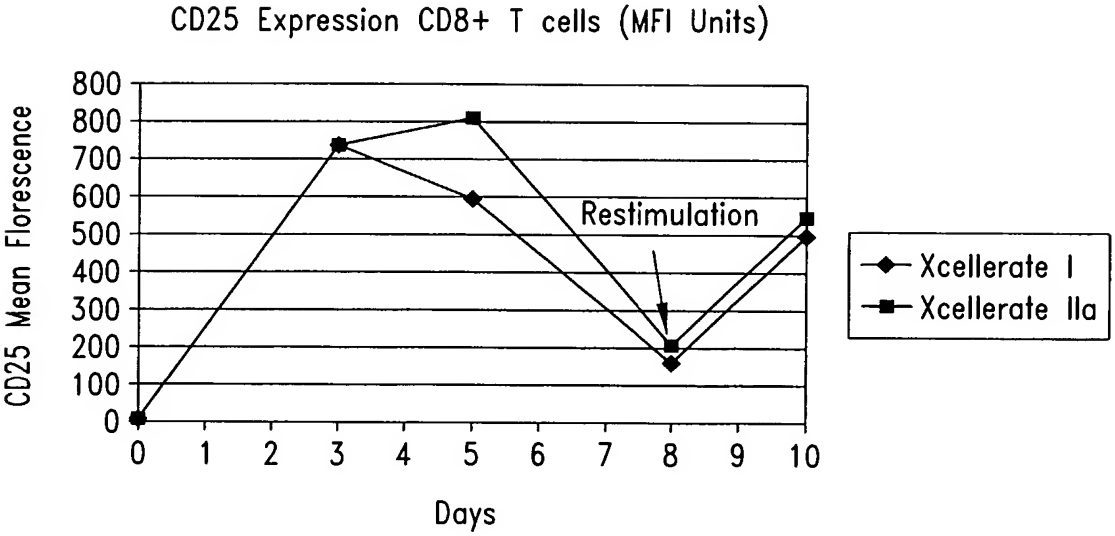

CD25 Expression CD8+ T cells (MFI Units)
CD25 Mean Florescence
800
800
700
600
500
400
300
200
100
0
0 1 2 3 4 5 6 7 8 9 10
Days
Restimulation
Xcellerate I
Xcellerate IIa

Fig. 6B

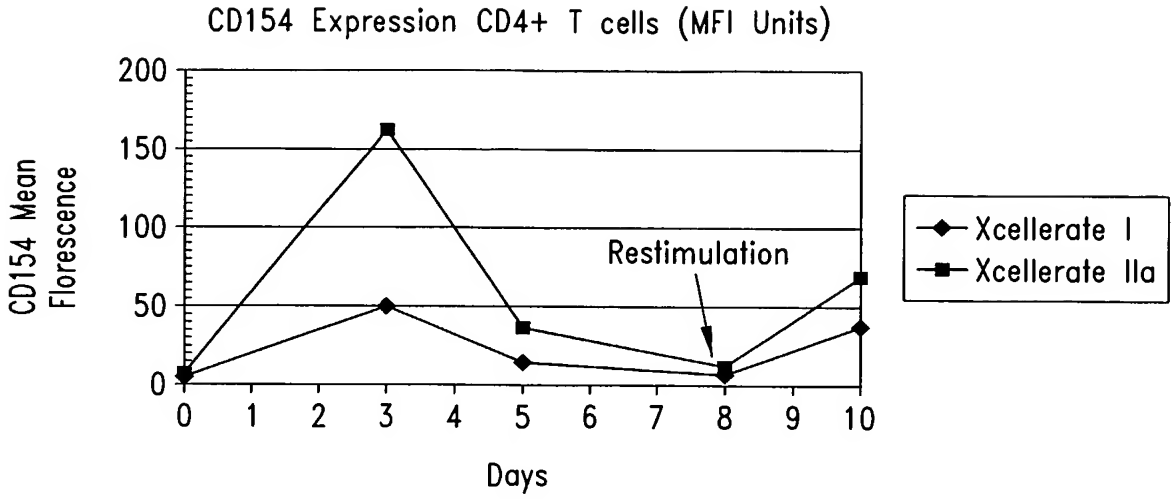

CD154 Expression CD4+ T cells (MFI Units)
CD154 Mean Florescence
200
150
100
50
0
0 1 2 3 4 5 6 7 8 9 10
Days
Restimulation
Xcellerate I
Xcellerate IIa

Fig. 7A

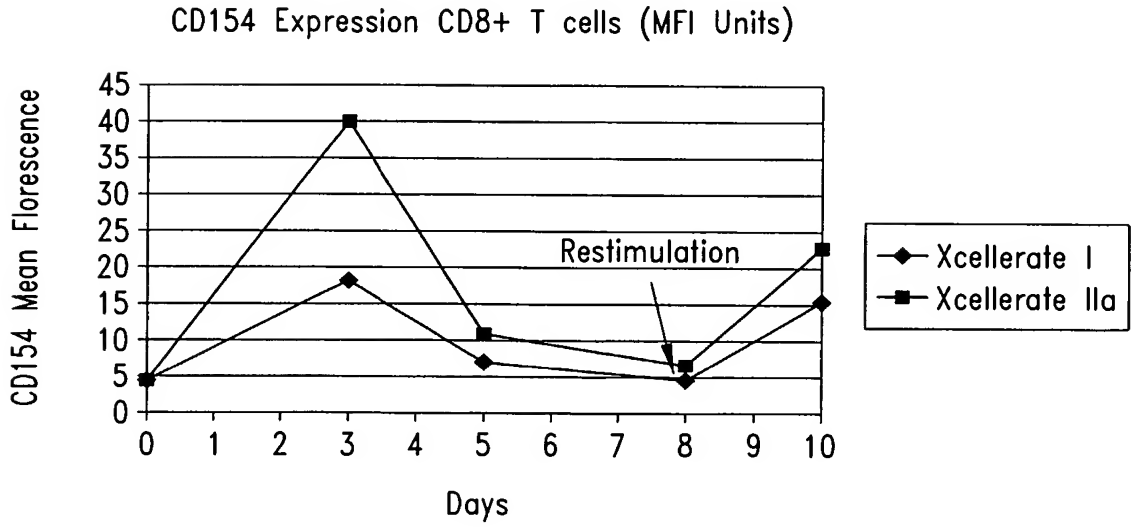

CD154 Expression CD8+ T cells (MFI Units)
CD154 Mean Florescence
45
40
35
30
25
20
15
10
5
0
0 1 2 3 4 5 6 7 8 9 10
Days
Restimulation
Xcellerate I
Xcellerate IIa

Fig. 7B

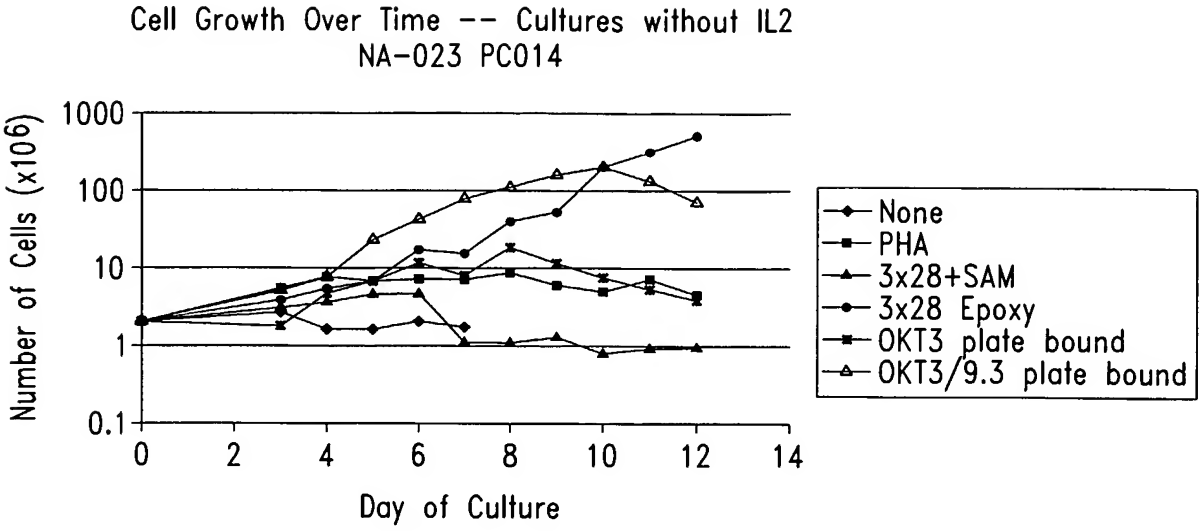

Cell Growth Over Time -- Cultures without IL2
NA-023 PC014
Number of Cells (x106)
1000
100
10
1
0.1
0
2
4
6
8
10
12
14
Day of Culture
None
PHA
3x28+SAM
3x28 Epoxy
OKT3 plate bound
OKT3/9.3 plate bound

Fig. 8A

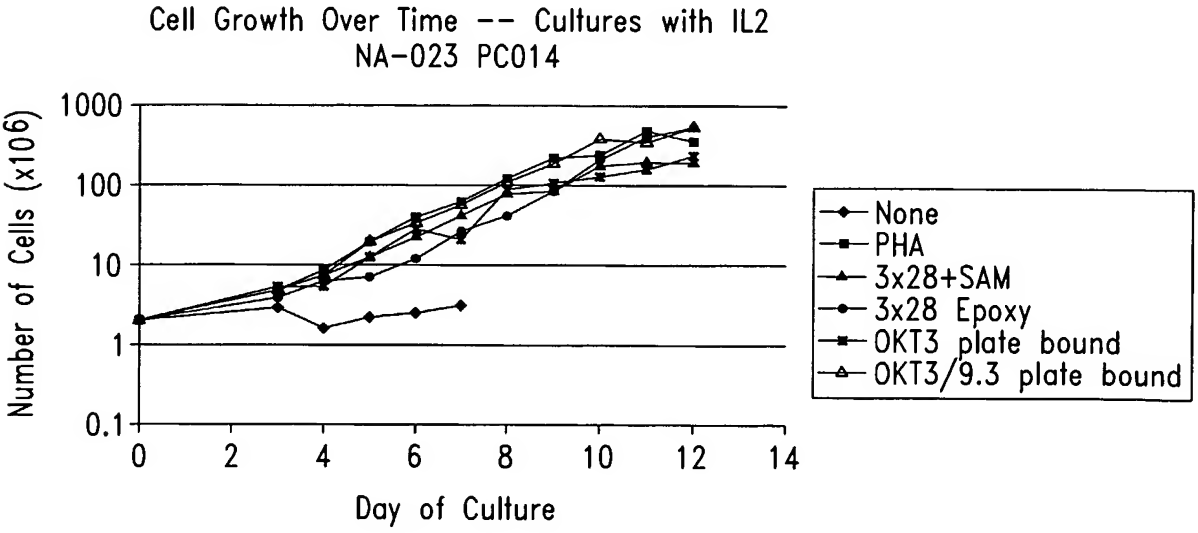

Cell Growth Over Time -- Cultures with IL2
NA-023 PC014
Number of Cells (x106)
1000
100
10
1
0.1
0
2
4
6
8
10
12
14
Day of Culture
None
PHA
3x28+SAM
3x28 Epoxy
OKT3 plate bound
OKT3/9.3 plate bound

Fig. 8B

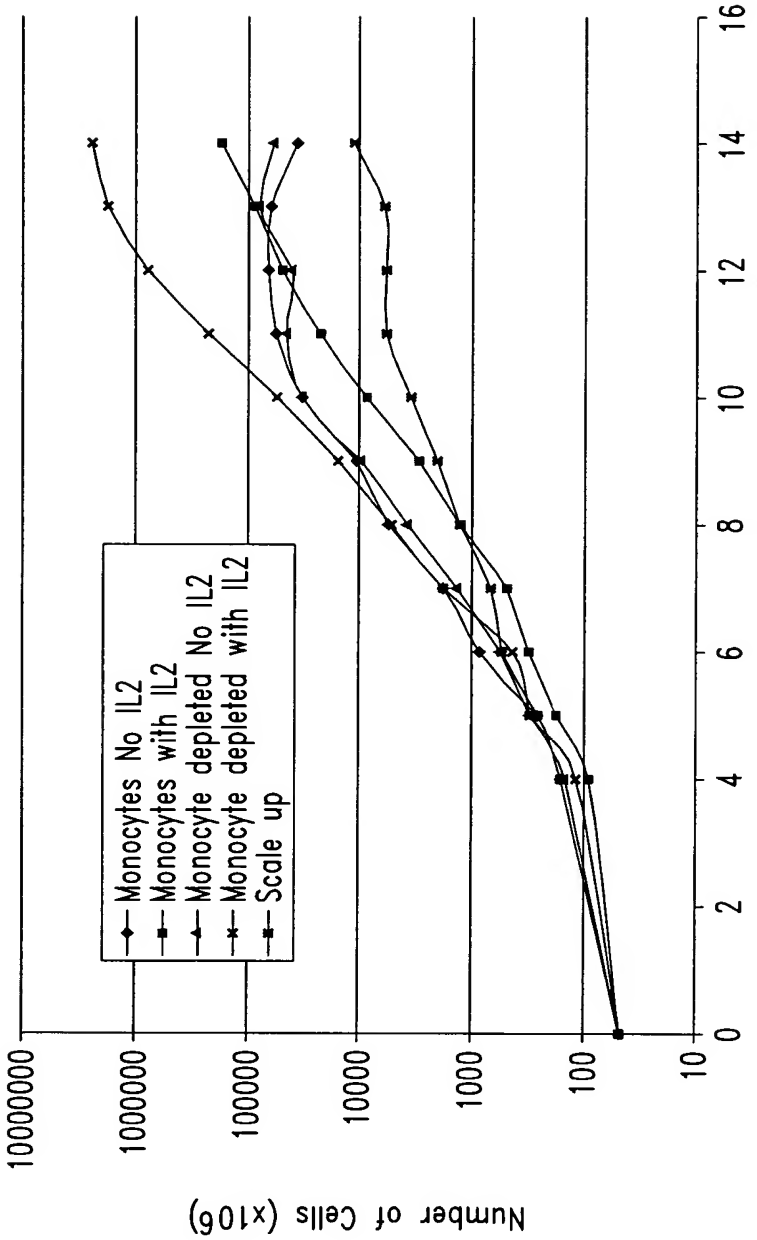

Monocytes No IL2
Monocytes with IL2
Monocyte depleted No IL2
Monocyte depleted with IL2
Scale up
Number of Cells (x10⁶)
10000000
1000000
100000
10000
1000
100
10
0
2
4
6
8
10
12
14
16
Day of Culture

Fig. 9

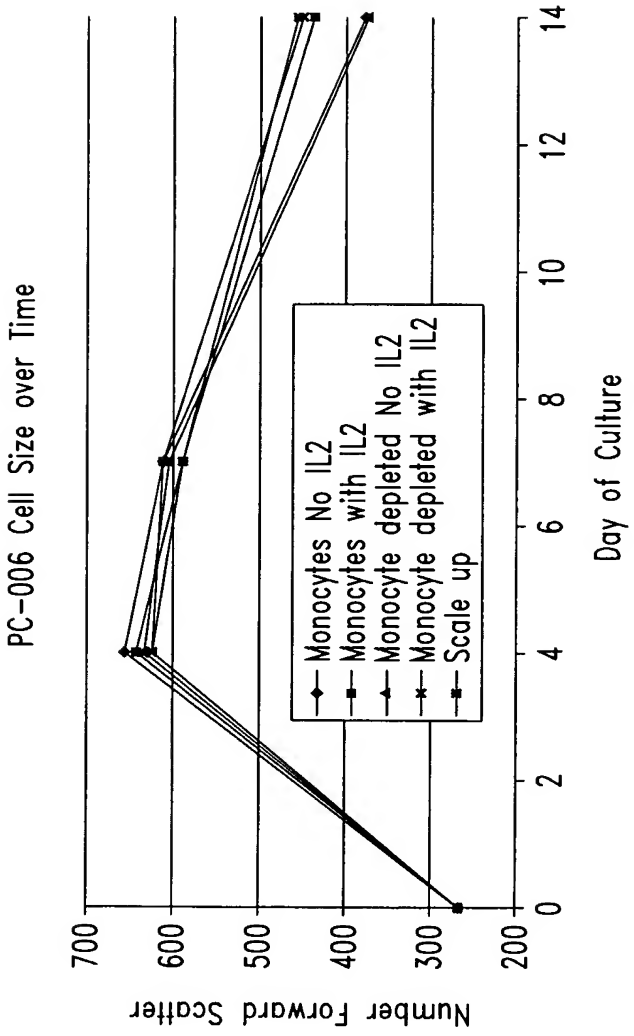

PC-006 Cell Size over Time
Number Forward Scatter
Day of Culture
700
600
500
400
300
200
0
2
4
6
8
10
12
14
Monocytes No IL2
Monocytes with IL2
Monocyte depleted No IL2
Monocyte depleted with IL2
Scale up

Fig. 10

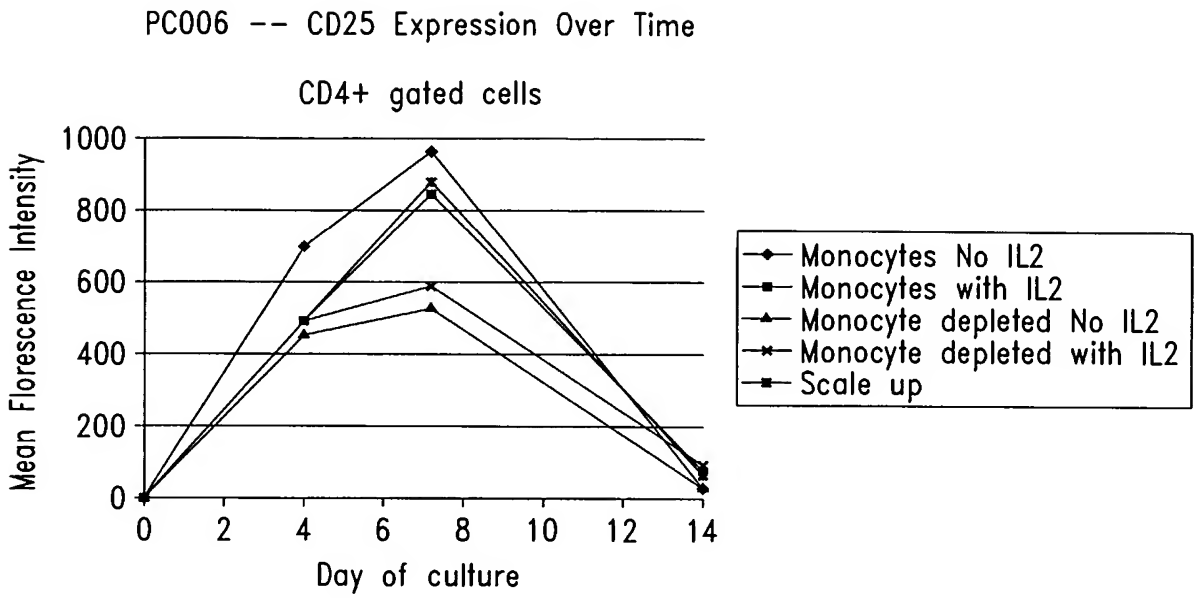


Fig. 11A

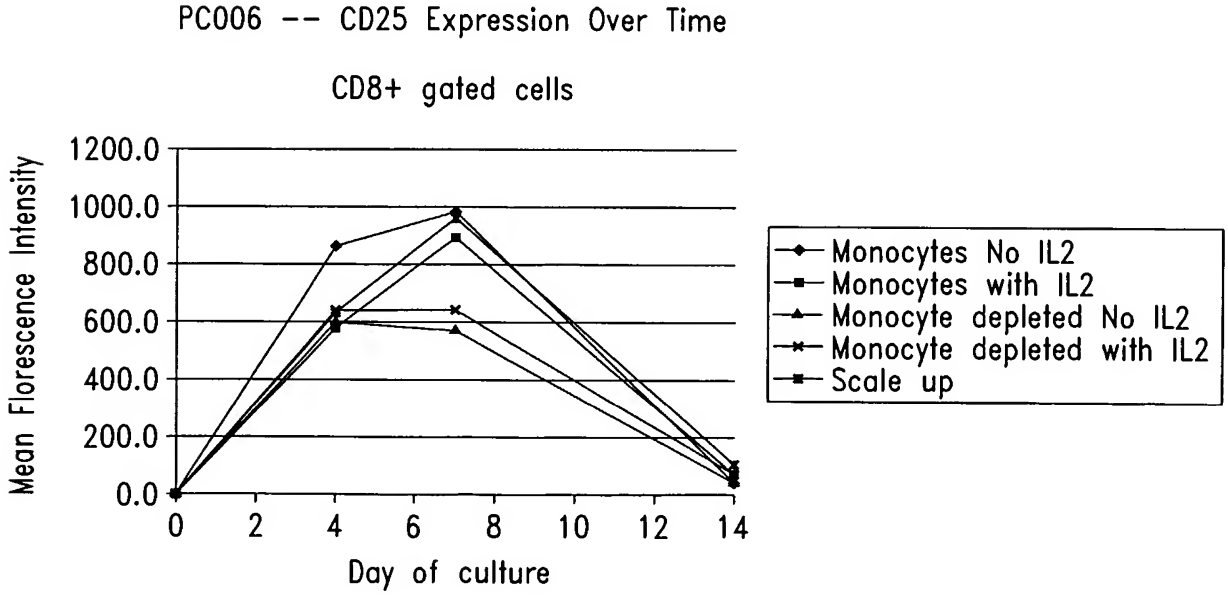


Fig. 11B

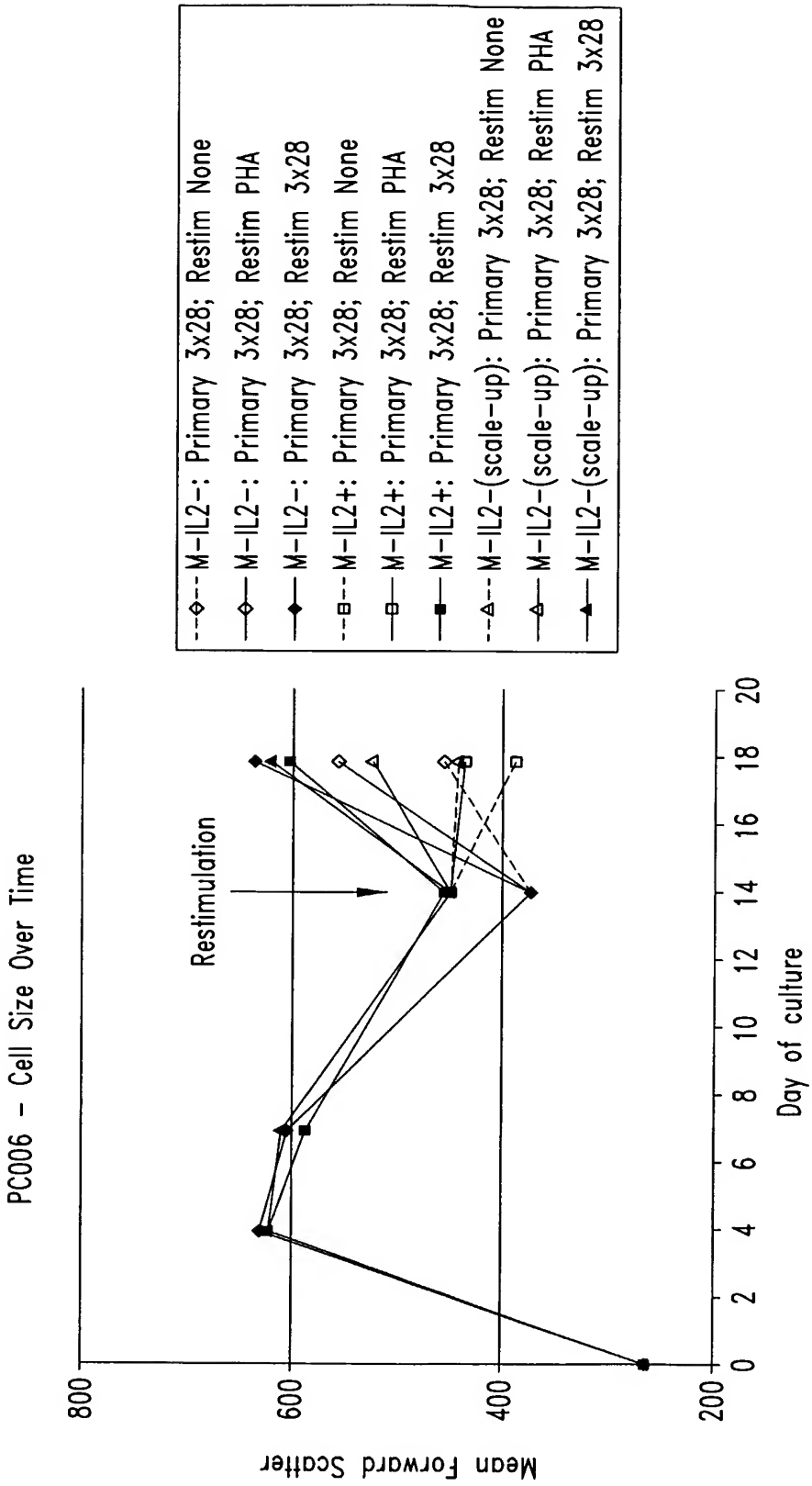

PC006 - Cell Size Over Time
Restimulation
Mean Forward Scatter
800
600
400
200
0
2
4
6
8
10
12
14
16
18
20
Day of culture
M-IL2-: Primary 3x28; Restim None
M-IL2-: Primary 3x28; Restim PHA
M-IL2-: Primary 3x28; Restim 3x28
M-IL2+: Primary 3x28; Restim None
M-IL2+: Primary 3x28; Restim PHA
M-IL2+: Primary 3x28; Restim 3x28
M-IL2-(scale-up): Primary 3x28; Restim None
M-IL2-(scale-up): Primary 3x28; Restim PHA
M-IL2-(scale-up): Primary 3x28; Restim 3x28

Fig. 12

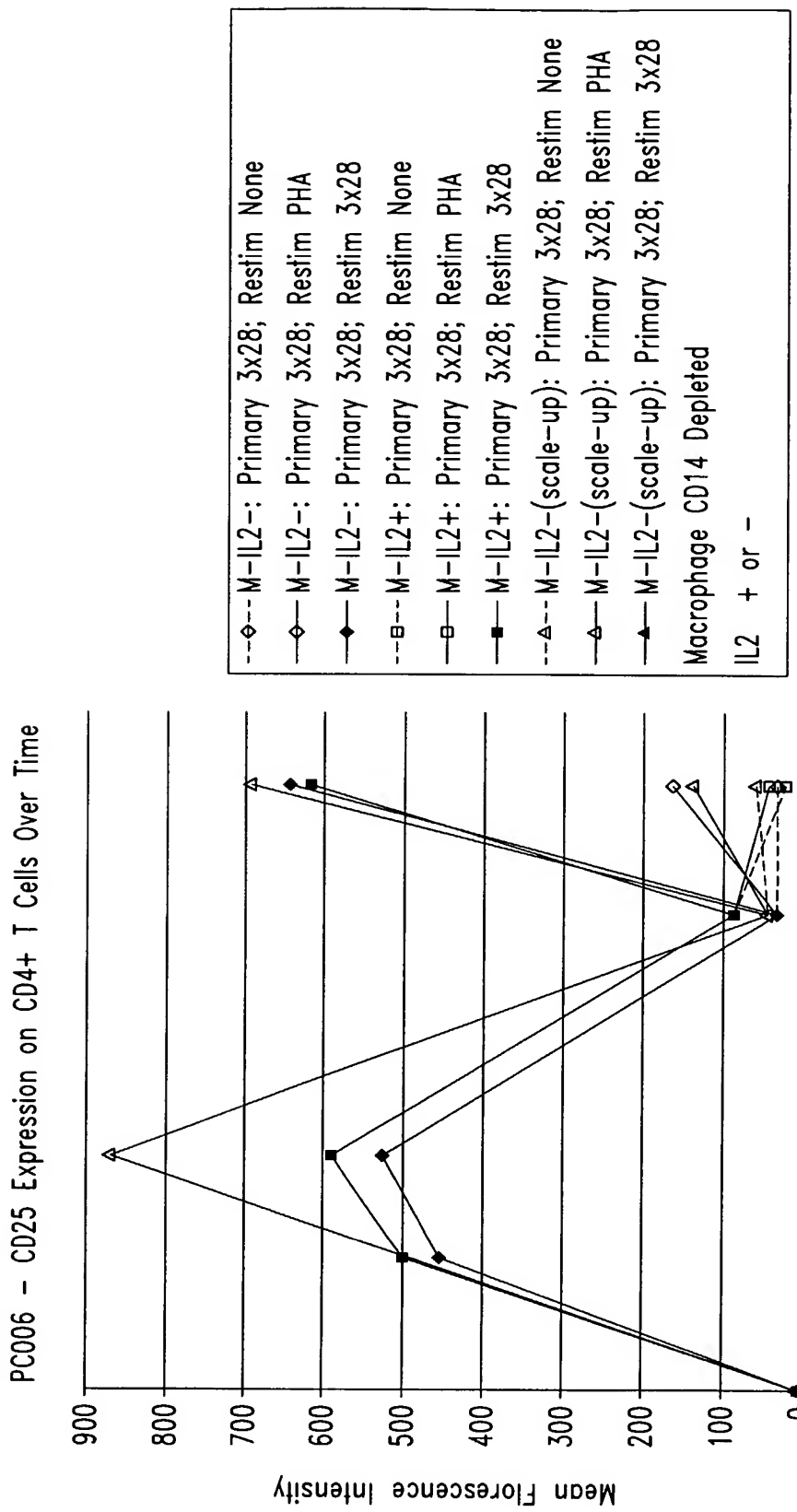

PC006 - CD25 Expression on CD4+ T Cells Over Time
Mean Florescence Intensity
Day of culture
0
100
200
300
400
500
600
700
800
900
0
2
4
6
8
10
12
14
16
18
20
M-IL2-: Primary 3x28; Restim None
M-IL2-: Primary 3x28; Restim PHA
M-IL2-: Primary 3x28; Restim 3x28
M-IL2+: Primary 3x28; Restim None
M-IL2+: Primary 3x28; Restim PHA
M-IL2+: Primary 3x28; Restim 3x28
M-IL2-(scale-up): Primary 3x28; Restim None
M-IL2-(scale-up): Primary 3x28; Restim PHA
M-IL2-(scale-up): Primary 3x28; Restim 3x28
Macrophage CD14 Depleted
IL2 + or -

Fig. 13A

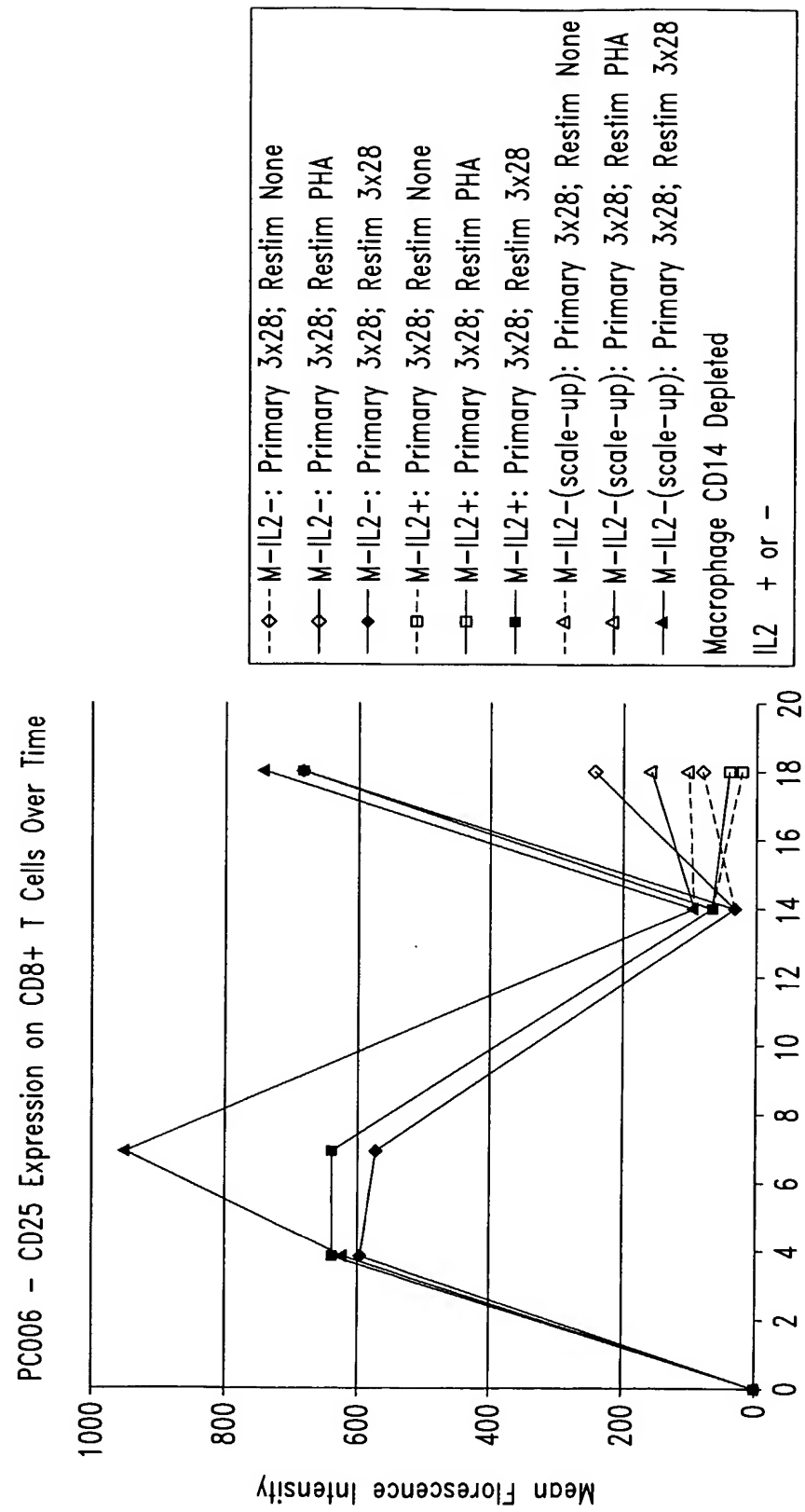

PC006 - CD25 Expression on CD8+ T Cells Over Time
Mean Florescence Intensity
1000
800
600
400
200
0
0
2
4
6
8
10
12
14
16
18
20
Day of culture
M-IL2-: Primary 3x28; Restim None
M-IL2-: Primary 3x28; Restim PHA
M-IL2-: Primary 3x28; Restim 3x28
M-IL2+: Primary 3x28; Restim None
M-IL2+: Primary 3x28; Restim PHA
M-IL2+: Primary 3x28; Restim 3x28
M-IL2-(scale-up): Primary 3x28; Restim None
M-IL2-(scale-up): Primary 3x28; Restim PHA
M-IL2-(scale-up): Primary 3x28; Restim 3x28
Macrophage CD14 Depleted
IL2 + or -

Fig. 13B

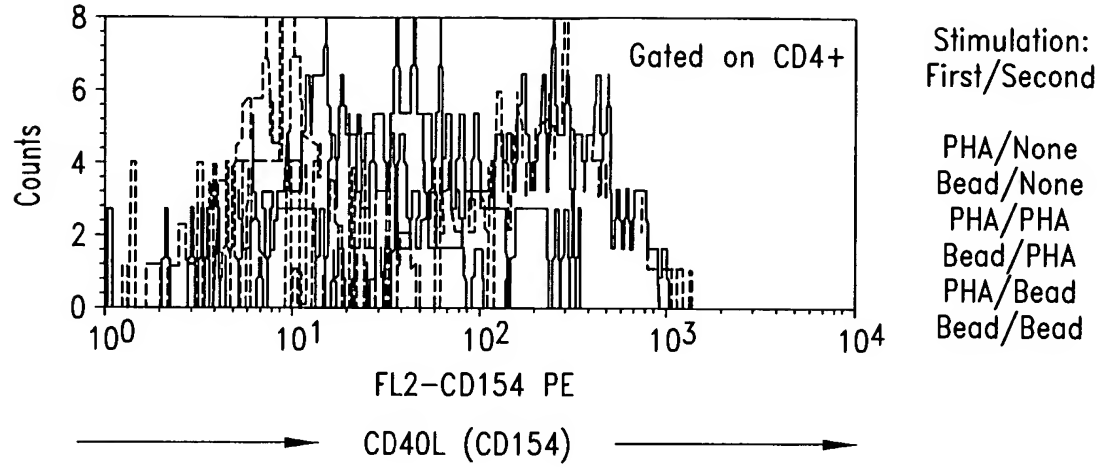


Fig. 14A

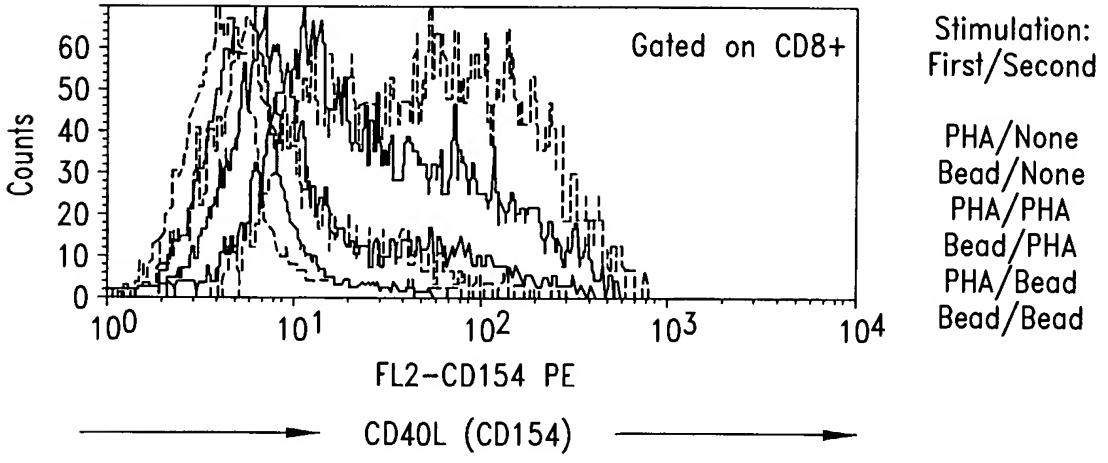


Fig. 14B

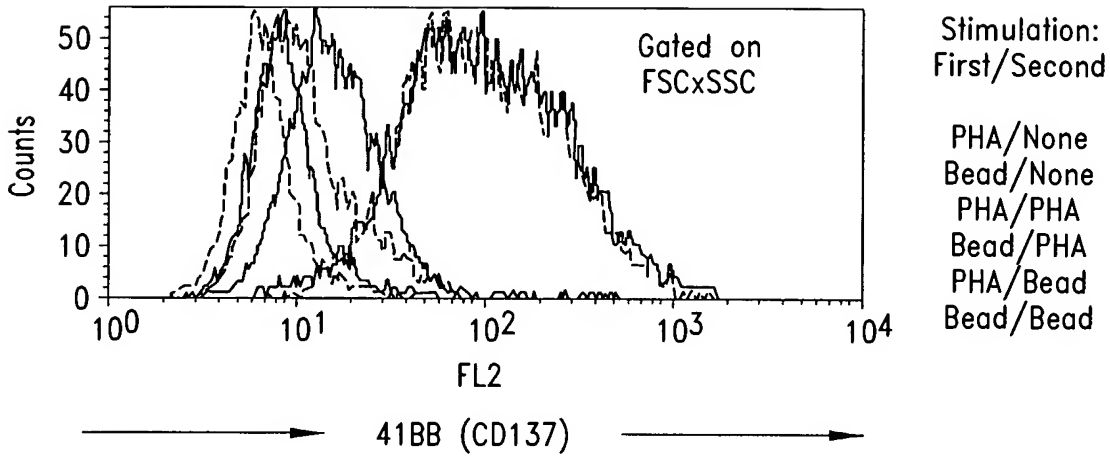

Counts
50
40
30
20
10
0
10^0
10^1
10^2
10^3
10^4
FL2
Gated on
FSCxSSC
41BB (CD137)
Stimulation:
First/Second
PHA/None
Bead/None
PHA/PHA
Bead/PHA
PHA/Bead
Bead/Bead

Fig. 15

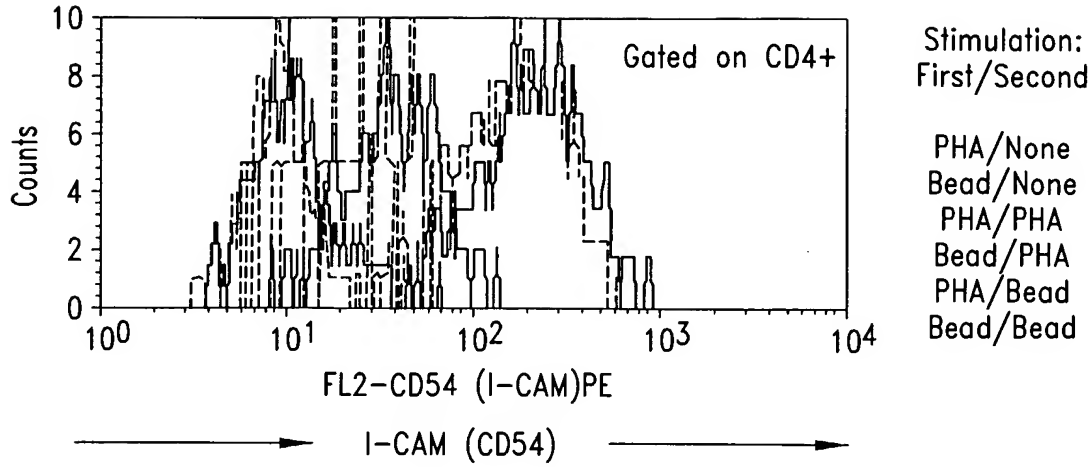


Fig. 16A

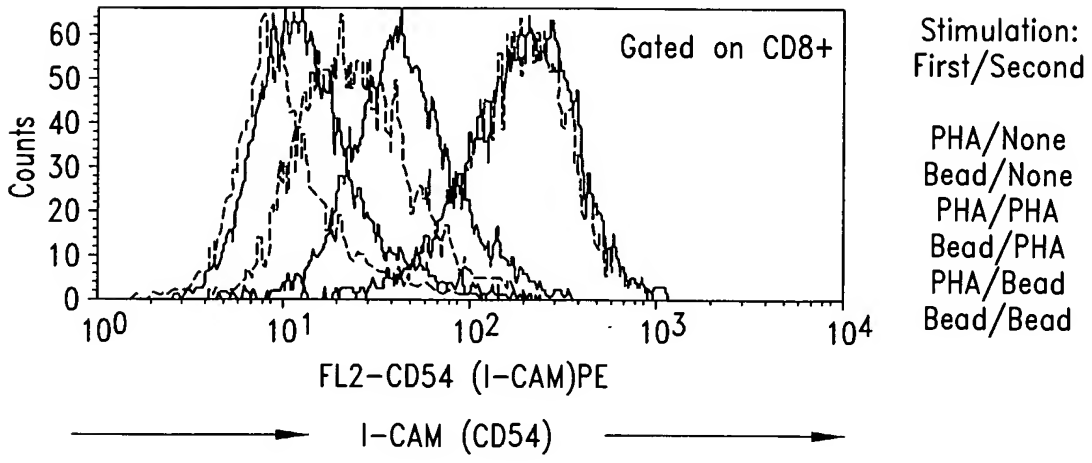


Fig. 16B

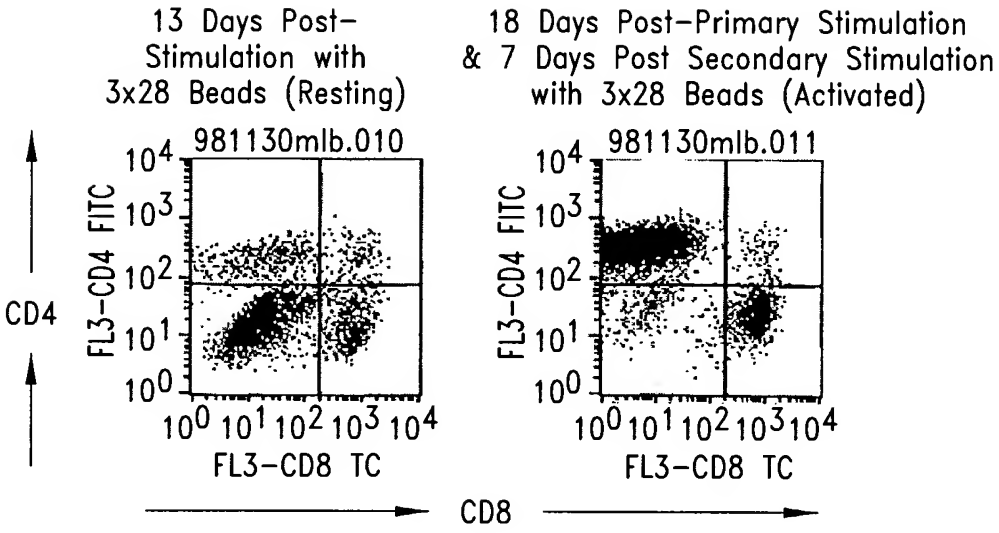


Fig. 17A *Fig. 17B*

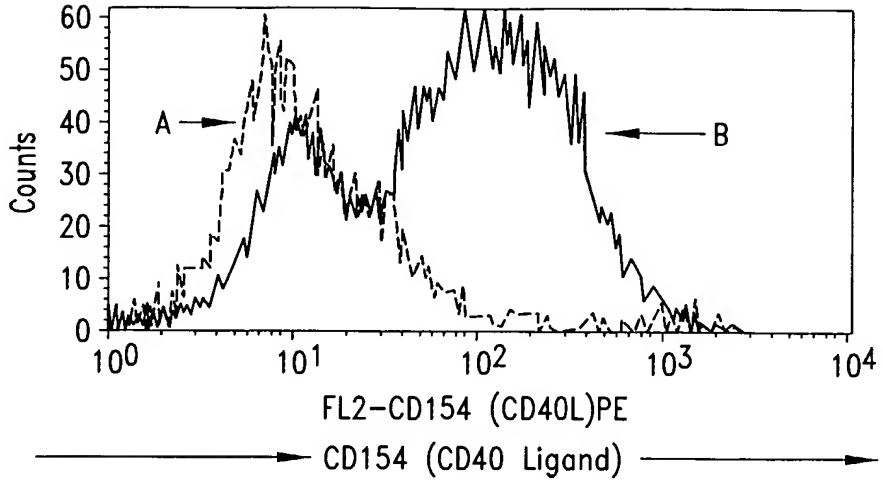


Fig. 17C

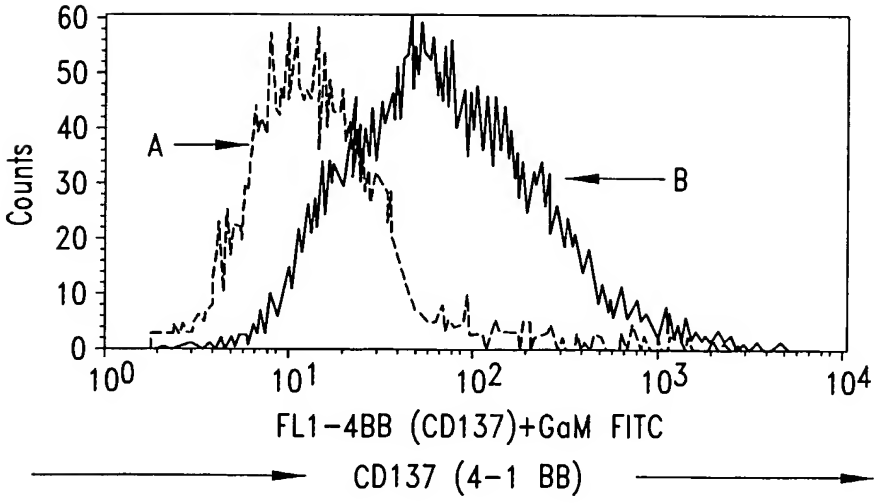


Fig. 17D

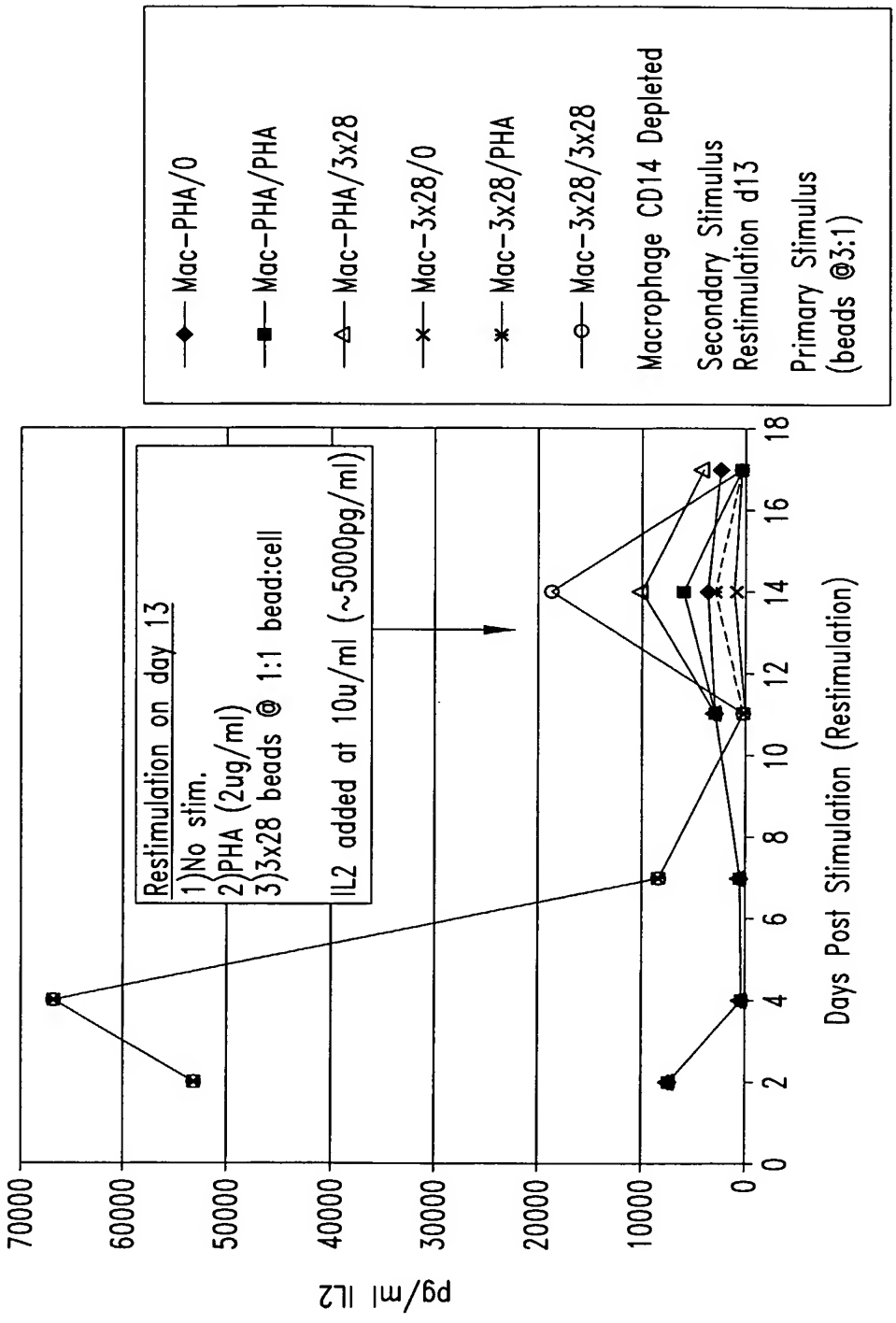

Mac-PHA/0
Mac-PHA/PHA
Mac-PHA/3x28
Mac-3x28/0
Mac-3x28/PHA
Mac-3x28/3x28
Macrophage CD14 Depleted
Secondary Stimulus
Restimulation d13
Primary Stimulus
(beads @3:1)
Restimulation on day 13
1)No stim.
2)PHA (2ug/ml)
3)3x28 beads @ 1:1 bead:cell
IL2 added at 10u/ml (~5000pg/ml)
pg/ml IL2
70000
60000
50000
40000
30000
20000
10000
0
0
2
4
6
8
10
12
14
16
18
Days Post Stimulation (Restimulation)

Fig. 18A

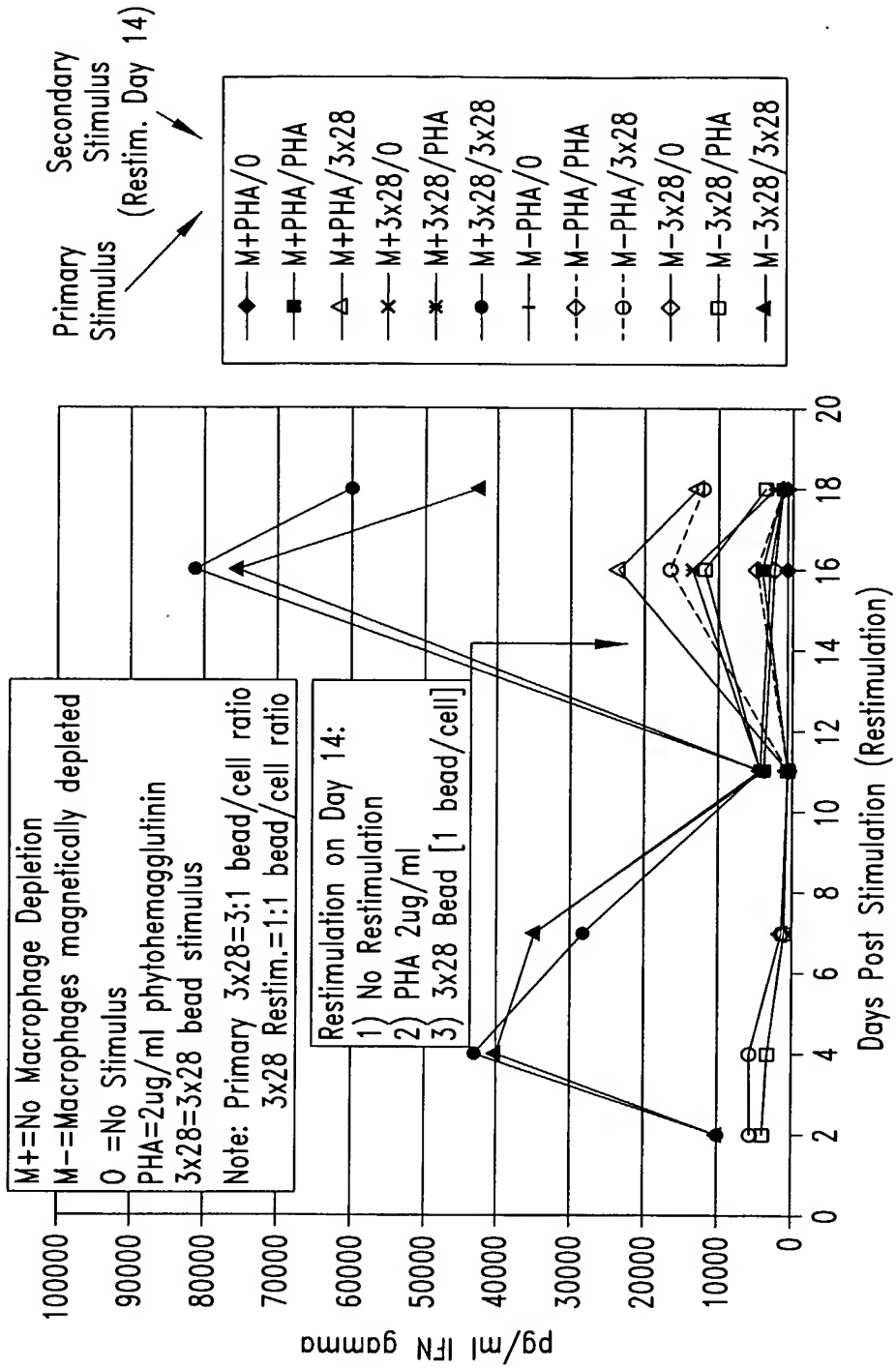

M+=No Macrophage Depletion
M-=Macrophages magnetically depleted
O =No Stimulus
PHA=2ug/ml phytohemagglutinin
3x28=3x28 bead stimulus
Note: Primary 3x28=3:1 bead/cell ratio
3x28 Restim.=1:1 bead/cell ratio
Restimulation on Day 14:
1) No Restimulation
2) PHA 2ug/ml
3) 3x28 Bead [1 bead/cell]
Primary Stimulus
Secondary Stimulus (Restim. Day 14)
M+PHA/0
M+PHA/PHA
M+PHA/3x28
M+3x28/0
M+3x28/PHA
M+3x28/3x28
M-PHA/0
M-PHA/PHA
M-PHA/3x28
M-3x28/0
M-3x28/PHA
M-3x28/3x28
pg/ml IFN gamma
Days Post Stimulation (Restimulation)

Fig. 18B

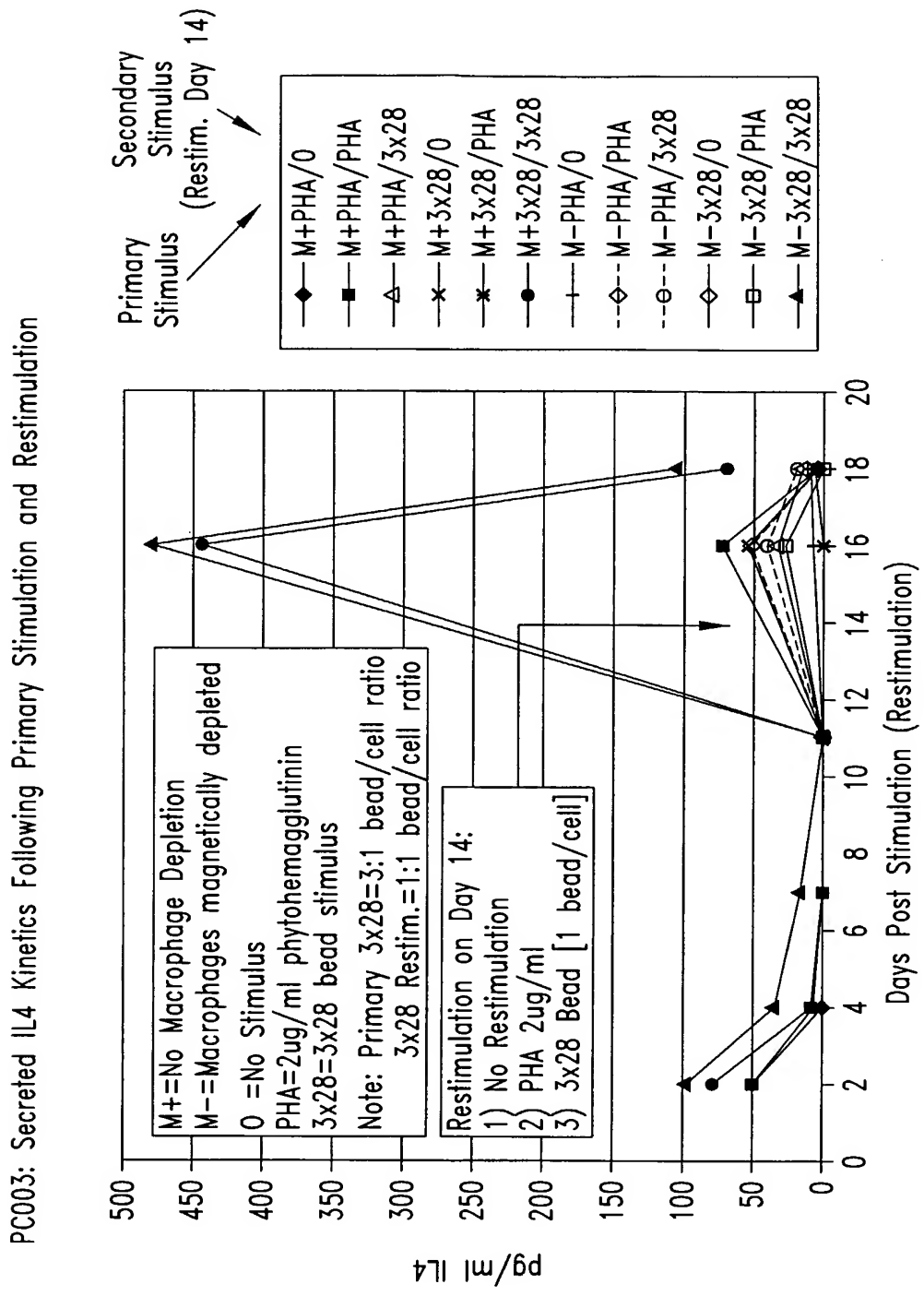

PC003: Secreted IL4 Kinetics Following Primary Stimulation and Restimulation
pg/ml IL4
Days Post Stimulation (Restimulation)
Primary Stimulus
Secondary Stimulus (Restim. Day 14)
M+=No Macrophage Depletion
M-=Macrophages magnetically depleted
O =No Stimulus
PHA=2ug/ml phytohemagglutinin
3x28=3x28 bead stimulus
Note: Primary 3x28=3:1 bead/cell ratio
3x28 Restim.=1:1 bead/cell ratio
Restimulation on Day 14:
1) No Restimulation
2) PHA 2ug/ml
3) 3x28 Bead [1 bead/cell]
M+PHA/0
M+PHA/PHA
M+PHA/3x28
M+3x28/0
M+3x28/PHA
M+3x28/3x28
M-PHA/0
M-PHA/PHA
M-PHA/3x28
M-3x28/0
M-3x28/PHA
M-3x28/3x28

Fig. 18C

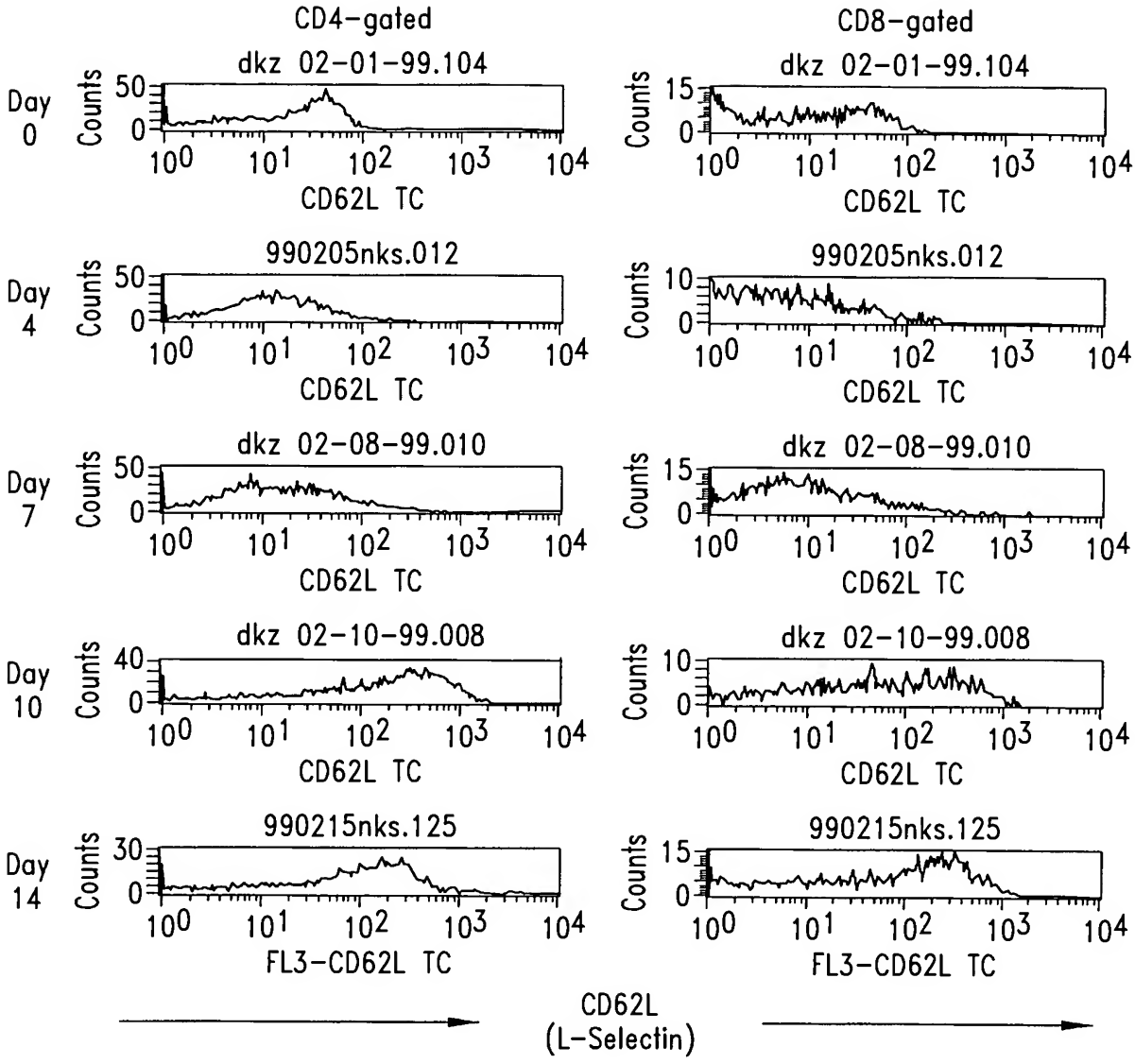

CD4-gated
CD8-gated
dkz 02-01-99.104
990205nks.012
dkz 02-08-99.010
dkz 02-10-99.008
990215nks.125
Day 0
Day 4
Day 7
Day 10
Day 14
Counts
CD62L TC
FL3-CD62L TC
CD62L
(L-Selectin)

Fig. 19A

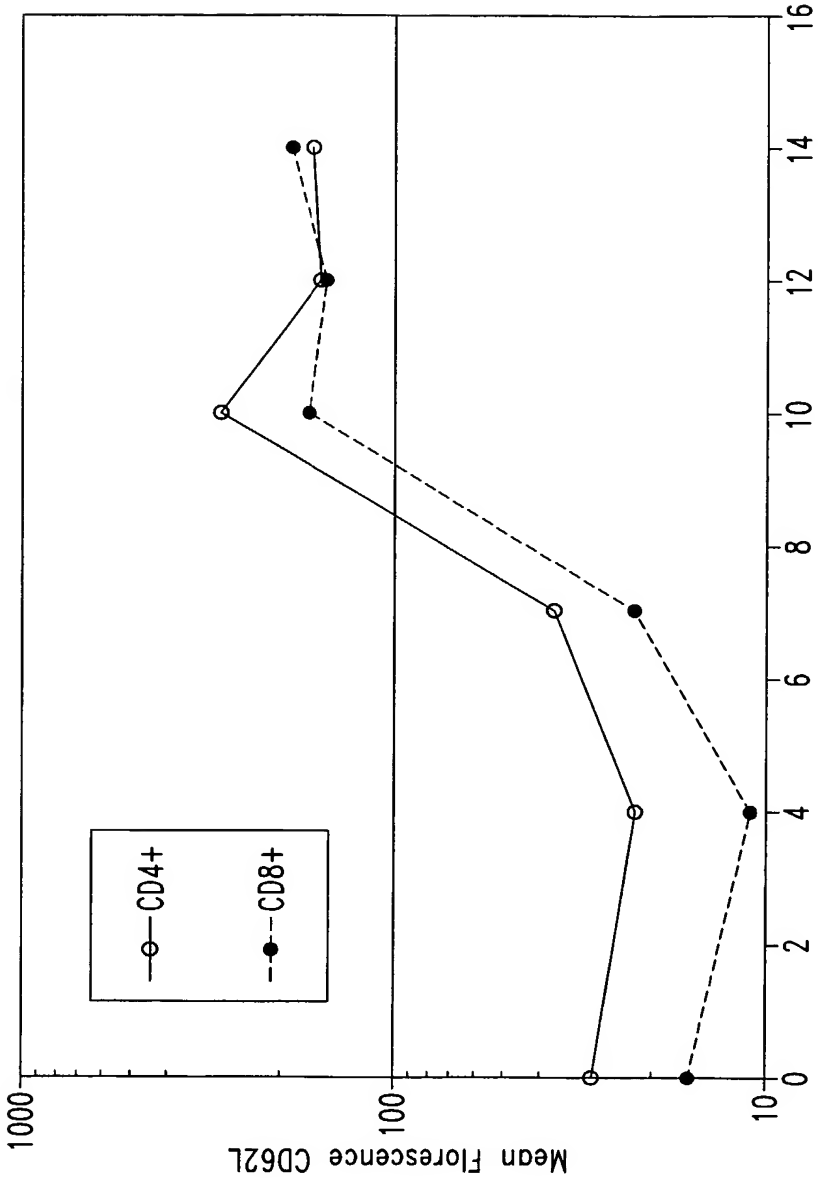

Mean Florescence CD62L
1000
100
10
0
2
4
6
8
10
12
14
16
Days Post-Activation
CD4+
CD8+

Fig. 19B

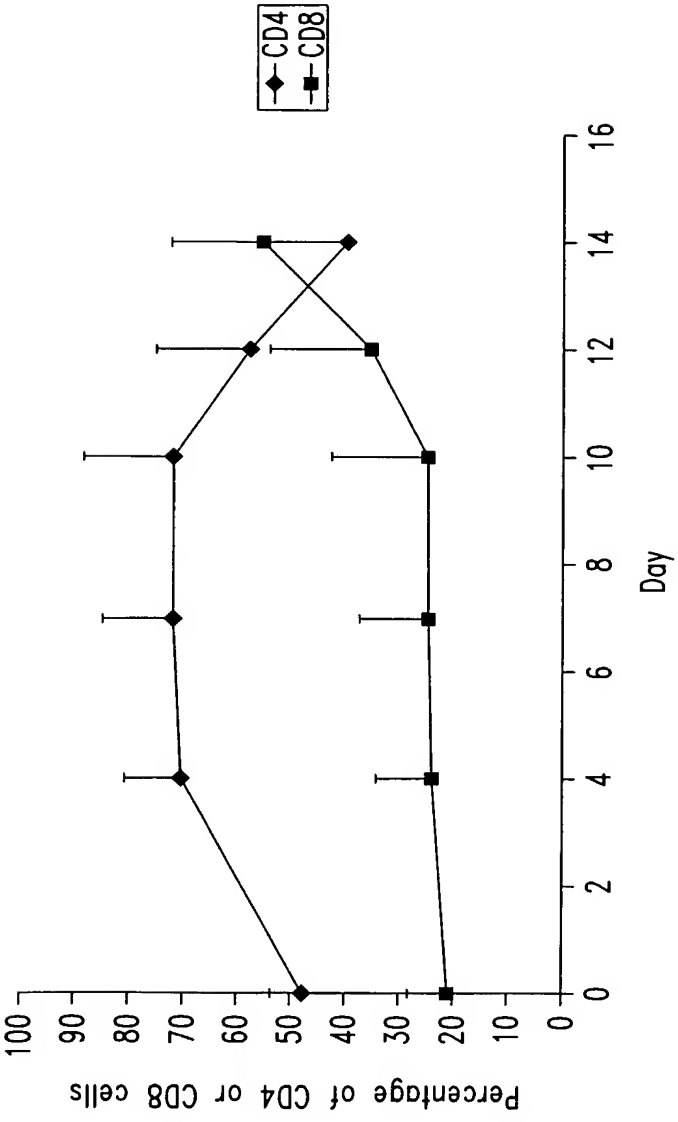

CD4
CD8
Percentage of CD4 or CD8 cells
100
90
80
70
60
50
40
30
20
10
0
0
2
4
6
8
10
12
14
16
Day

Fig. 20

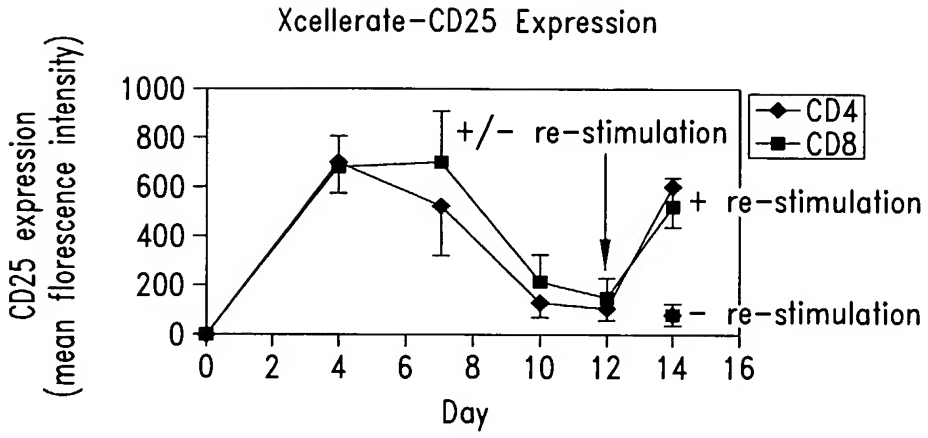


Fig. 21A

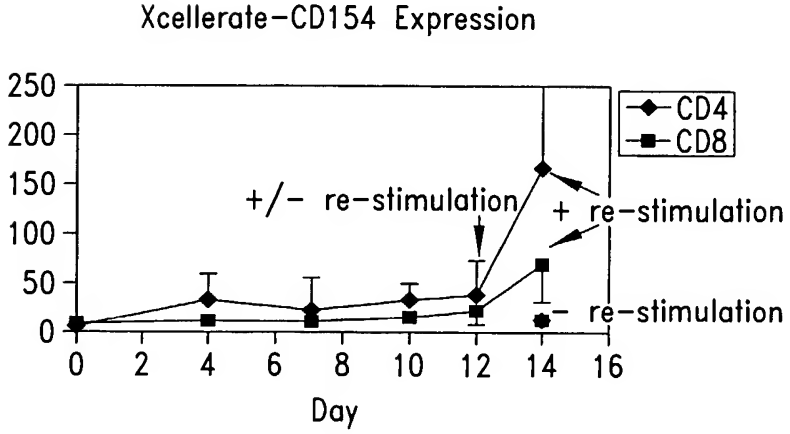


Fig. 21B

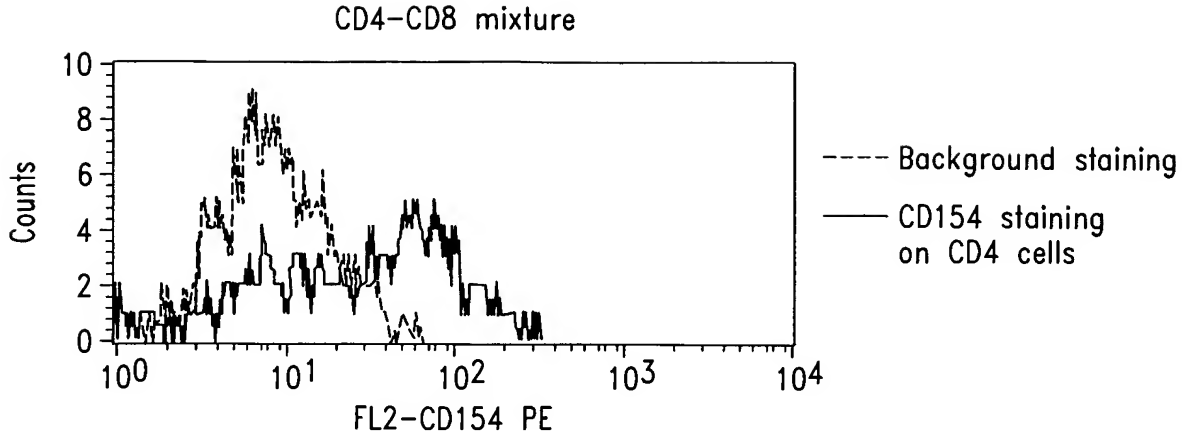


Fig. 22A

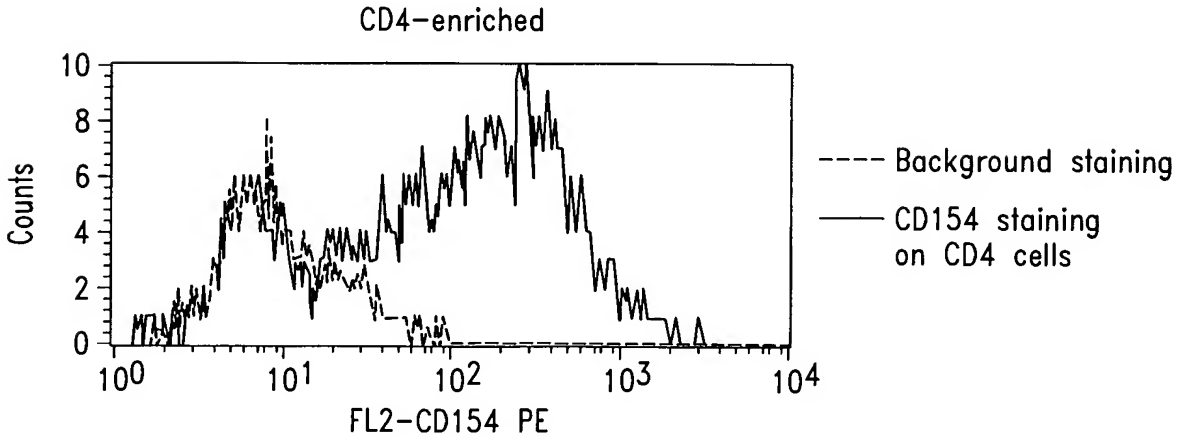


Fig. 22B

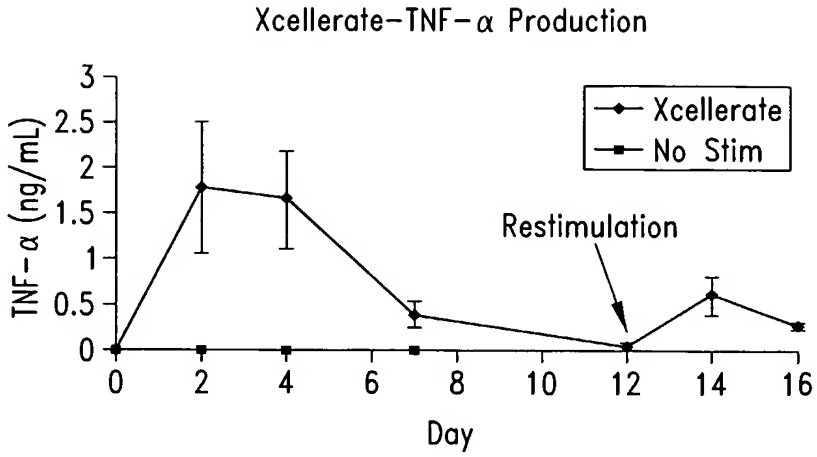


Fig. 23A

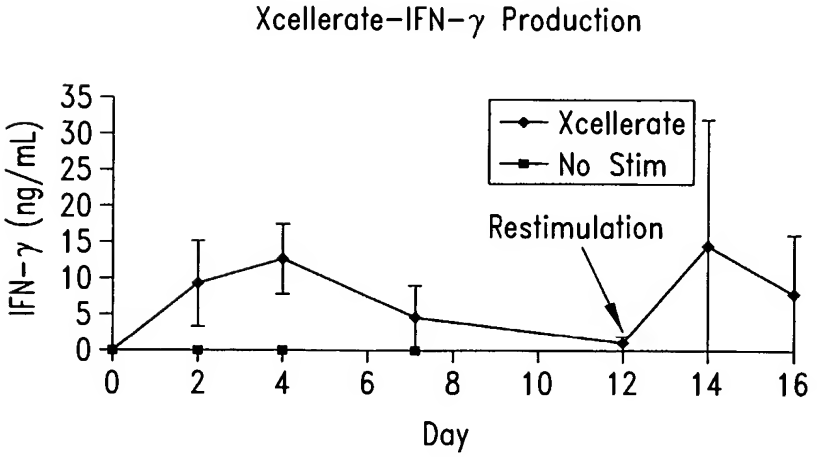


Fig. 23B

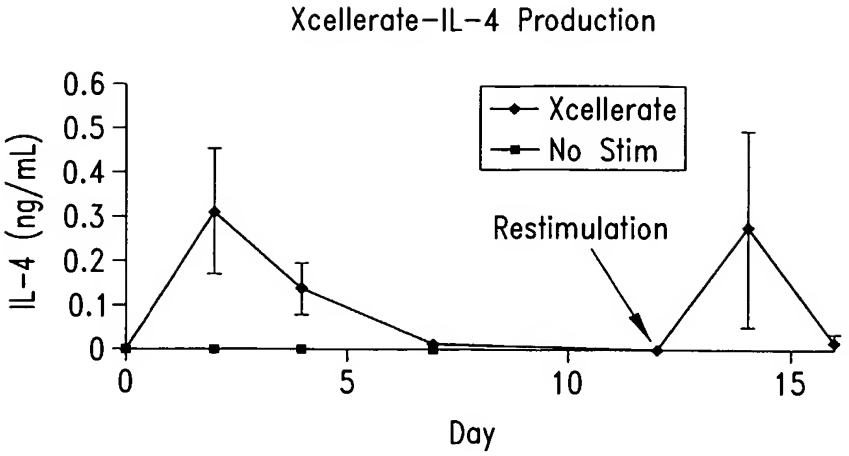


Fig. 24A

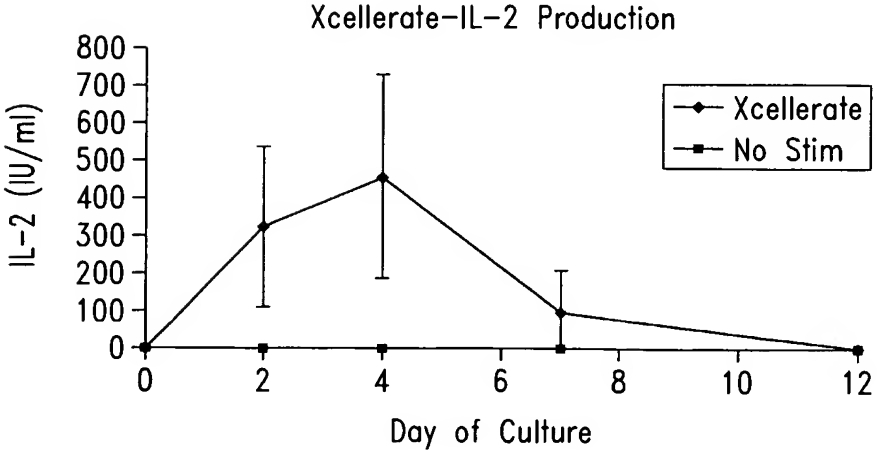


Fig. 24B

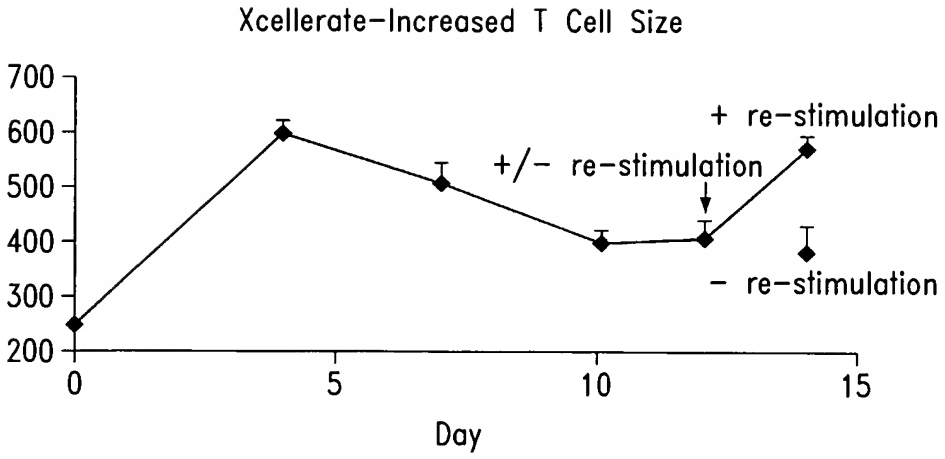
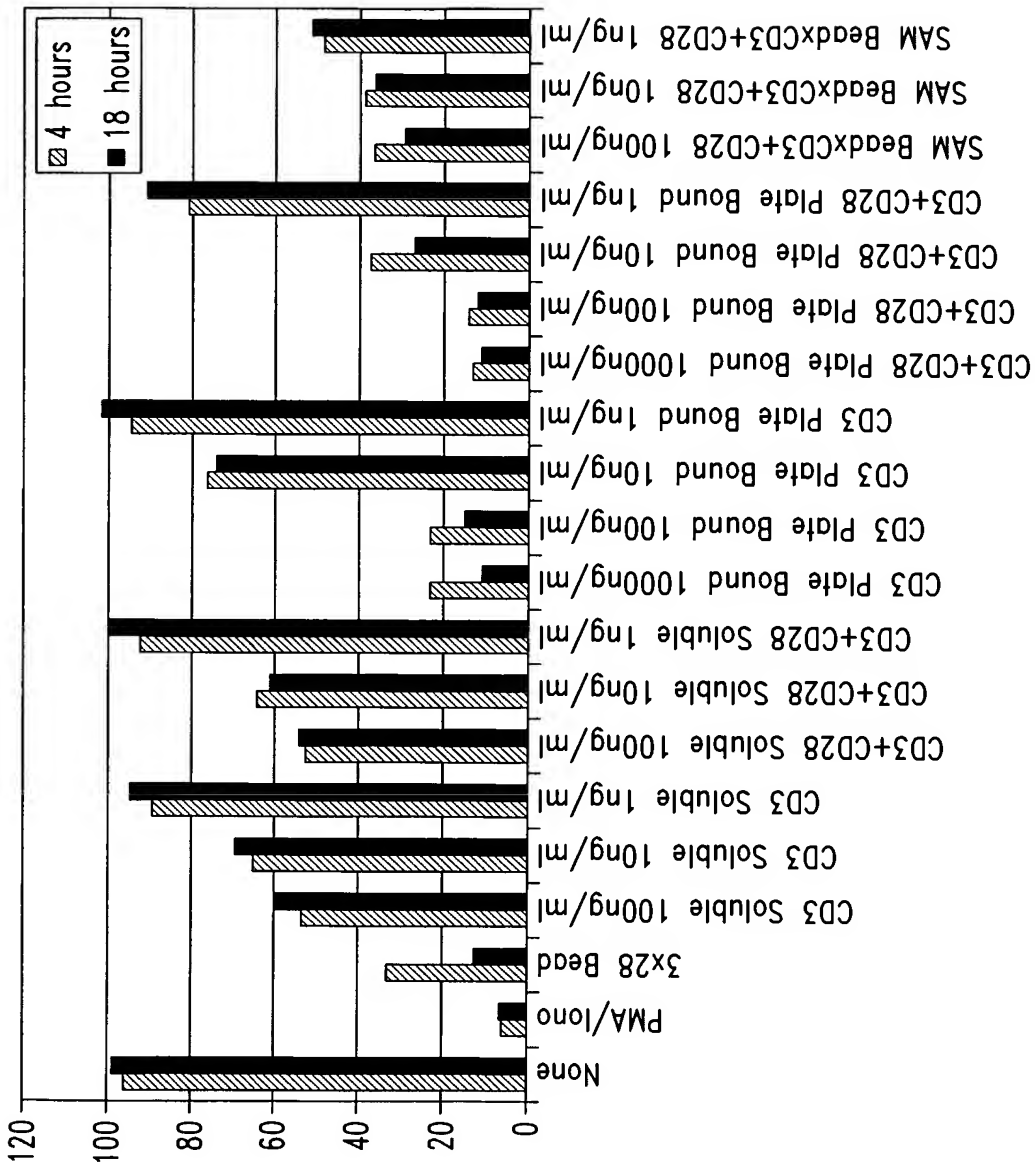

Xcellerate-Increased T Cell Size
700
600
500
400
300
200
0
5
10
15
Day
+ re-stimulation
+/- re-stimulation
- re-stimulation

Fig. 25



Restimulation of NDa055 (Day8):
CD62L (L-Selectin) Expression on CD4+ T Cells

Fig. 26A

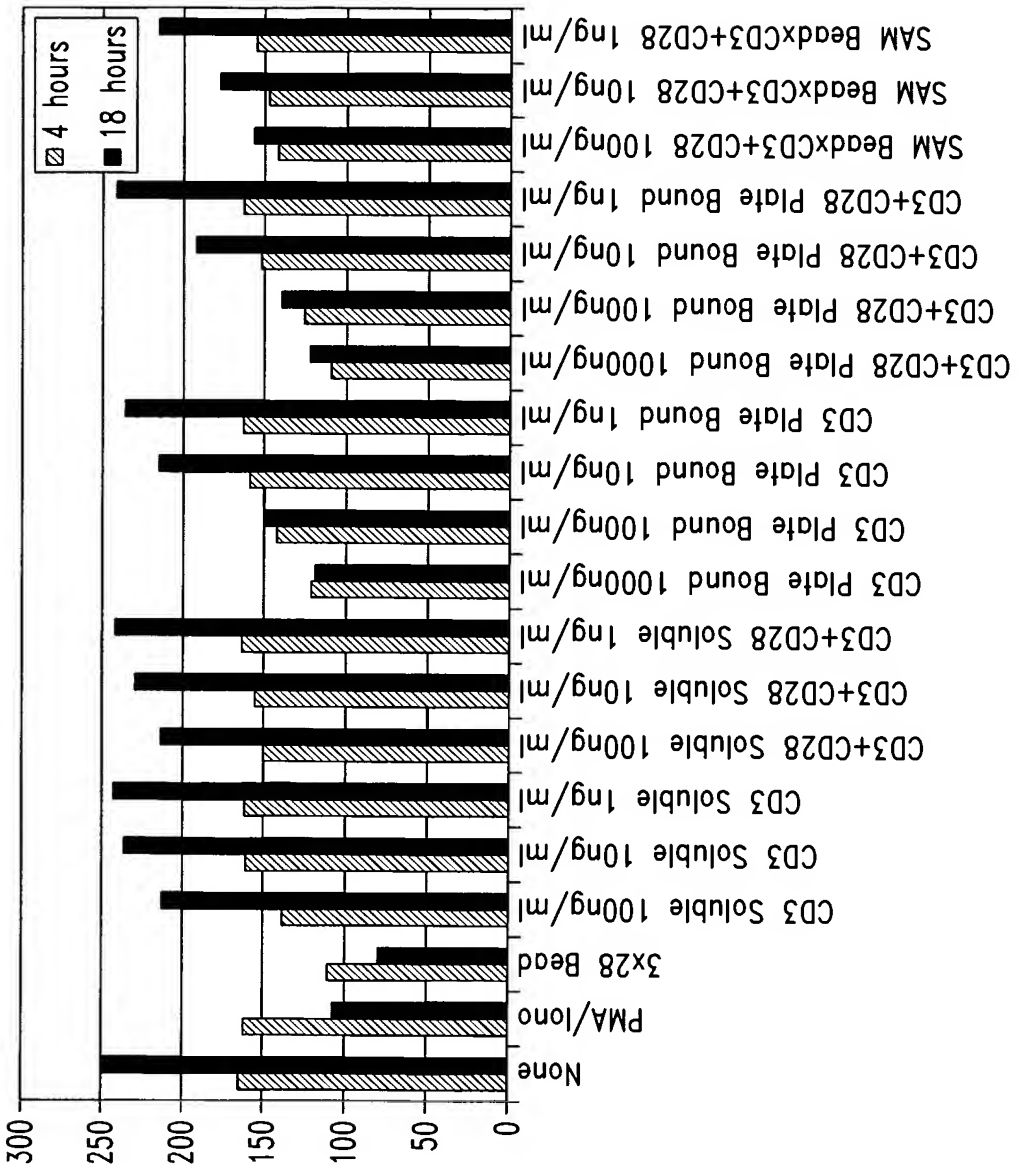

4 hours
18 hours
300
250
200
150
100
50
0
None
PMA/Iono
3x28 Bead
CD3 Soluble 100ng/ml
CD3 Soluble 10ng/ml
CD3 Soluble 1ng/ml
CD3+CD28 Soluble 100ng/ml
CD3+CD28 Soluble 10ng/ml
CD3+CD28 Soluble 1ng/ml
CD3 Plate Bound 1000ng/ml
CD3 Plate Bound 100ng/ml
CD3 Plate Bound 10ng/ml
CD3 Plate Bound 1ng/ml
CD3+CD28 Plate Bound 1000ng/ml
CD3+CD28 Plate Bound 100ng/ml
CD3+CD28 Plate Bound 10ng/ml
CD3+CD28 Plate Bound 1ng/ml
SAM BeadxCD3+CD28 100ng/ml
SAM BeadxCD3+CD28 10ng/ml
SAM BeadxCD3+CD28 1ng/ml
Restimulation of NDa055 (Day8):
CD49d (VLA4) Expression on CD4+ T Cells

Fig. 26B

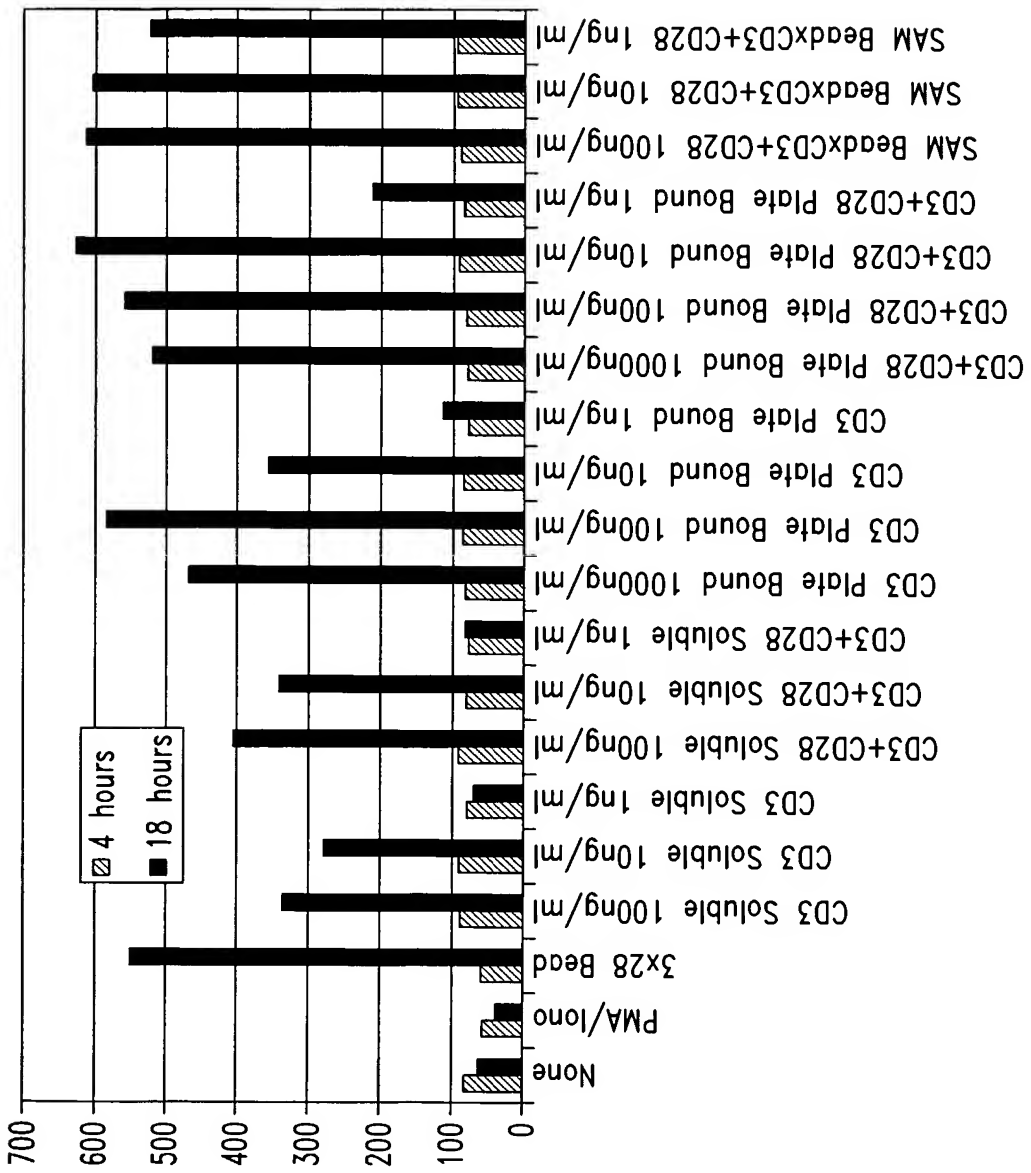
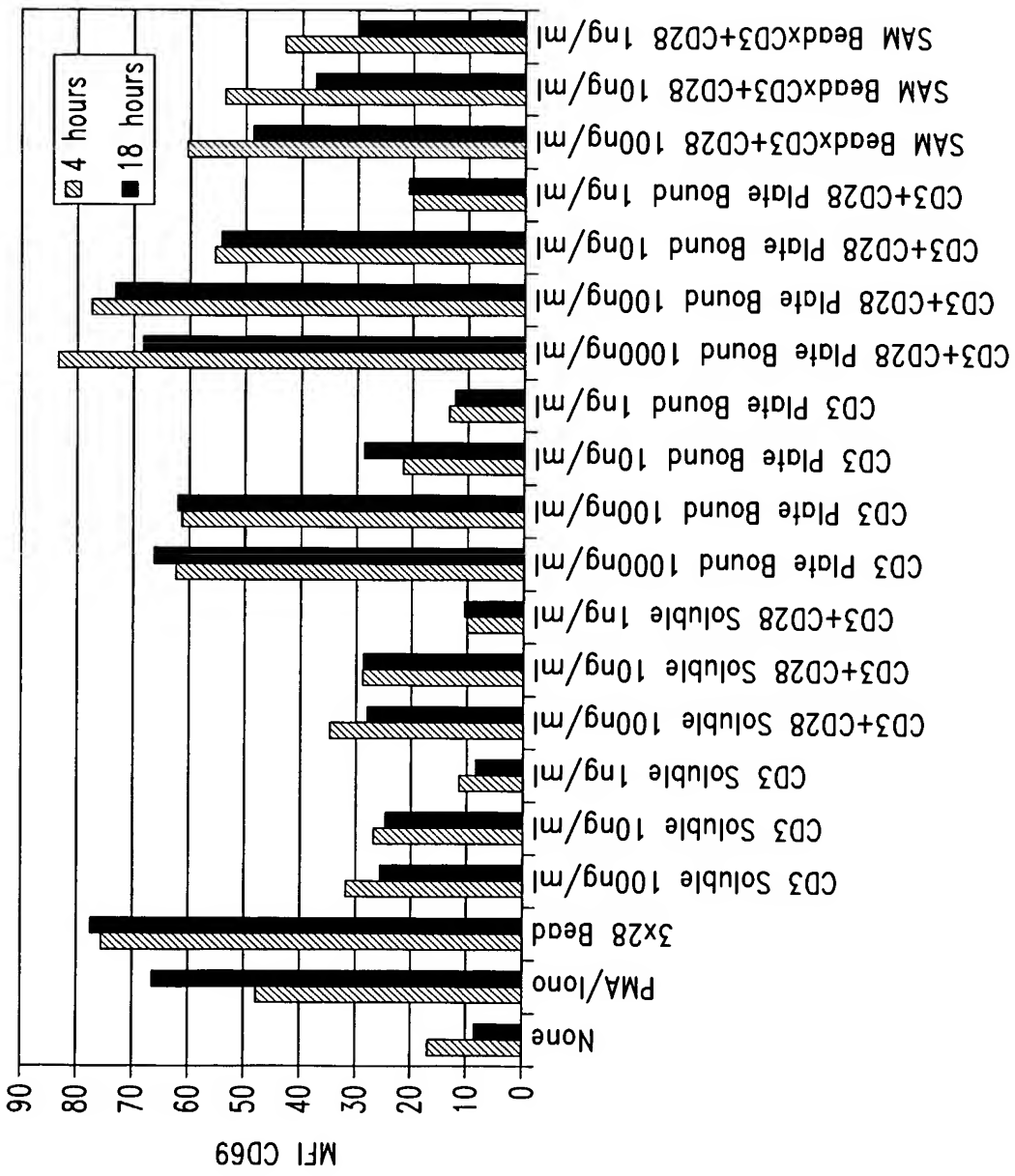
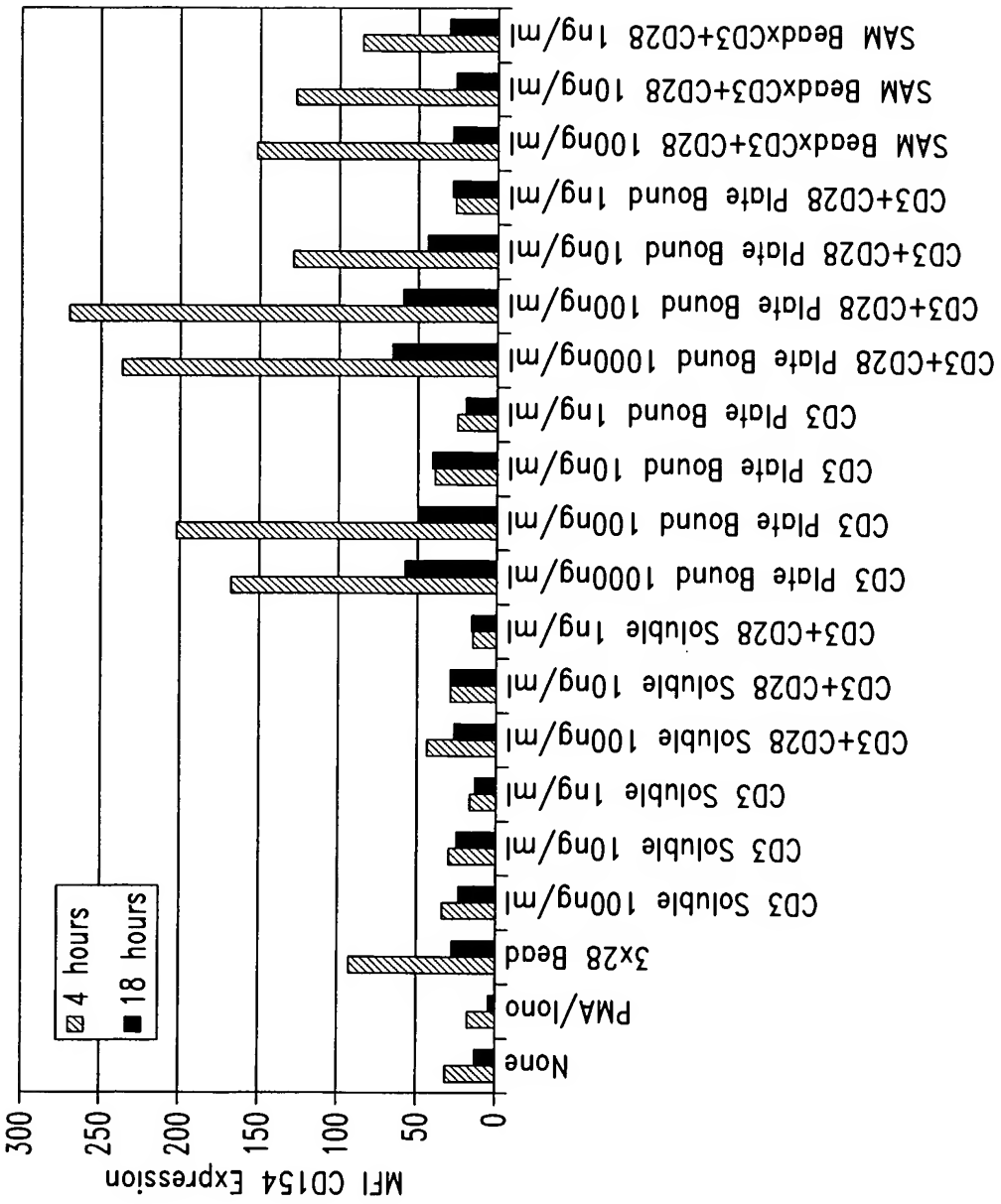

Restimulation of NDa055 (Day8):
CD25 (IL2-R) Expression on CD4+ T Cells
4 hours
18 hours
0
100
200
300
400
500
600
700
None
PMA/Iono
3x28 Bead
CD3 Soluble 100ng/ml
CD3 Soluble 10ng/ml
CD3 Soluble 1ng/ml
CD3+CD28 Soluble 100ng/ml
CD3+CD28 Soluble 10ng/ml
CD3+CD28 Soluble 1ng/ml
CD3 Plate Bound 1000ng/ml
CD3 Plate Bound 100ng/ml
CD3 Plate Bound 10ng/ml
CD3 Plate Bound 1ng/ml
CD3+CD28 Plate Bound 1000ng/ml
CD3+CD28 Plate Bound 100ng/ml
CD3+CD28 Plate Bound 10ng/ml
CD3+CD28 Plate Bound 1ng/ml
SAM BeadxCD3+CD28 100ng/ml
SAM BeadxCD3+CD28 10ng/ml
SAM BeadxCD3+CD28 1ng/ml

Fig. 26C



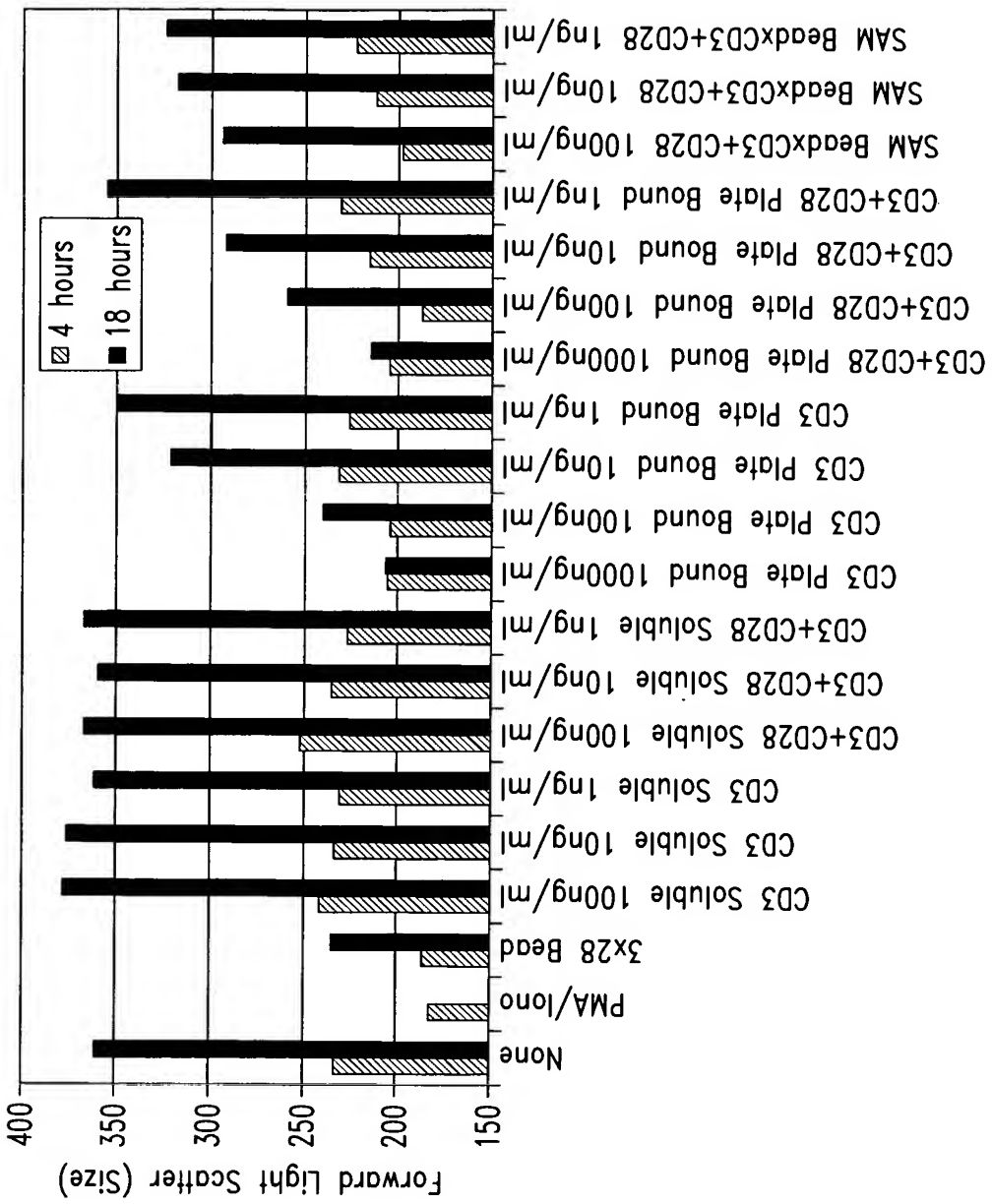
Restimulation of NDa055 (Day8):
CD69 Expression on CD4+ T Cells

Fig. 26D



Restimulation of NDa055 (Day8):
CD154 (CD40L) Expression on CD4+ T Cells

Fig. 26E



Restimulation of NDa055 (Day8):
Forward Light Scatter (Size)

Fig. 26F

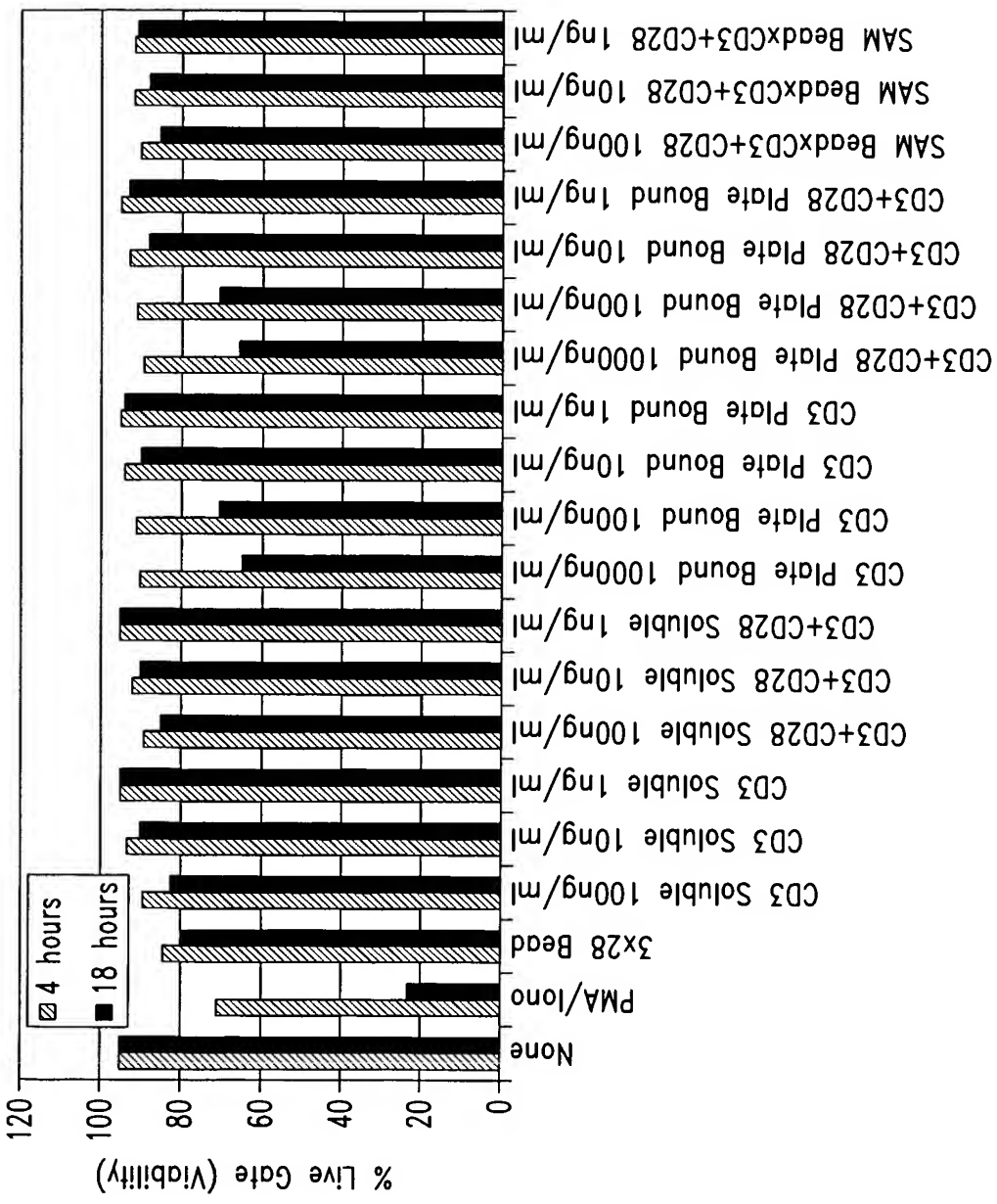

Restimulation of NDa055 (Day8):
Live Gate (Viability)
% Live Gate (Viability)
120
100
80
60
40
20
0
4 hours
18 hours
None
PMA/Iono
3x28 Bead
CD3 Soluble 100ng/ml
CD3 Soluble 10ng/ml
CD3 Soluble 1ng/ml
CD3+CD28 Soluble 100ng/ml
CD3+CD28 Soluble 10ng/ml
CD3+CD28 Soluble 1ng/ml
CD3 Plate Bound 1000ng/ml
CD3 Plate Bound 100ng/ml
CD3 Plate Bound 10ng/ml
CD3 Plate Bound 1ng/ml
CD3+CD28 Plate Bound 1000ng/ml
CD3+CD28 Plate Bound 100ng/ml
CD3+CD28 Plate Bound 10ng/ml
CD3+CD28 Plate Bound 1ng/ml
SAM BeadxCD3+CD28 100ng/ml
SAM BeadxCD3+CD28 10ng/ml
SAM BeadxCD3+CD28 1ng/ml

Fig. 26G

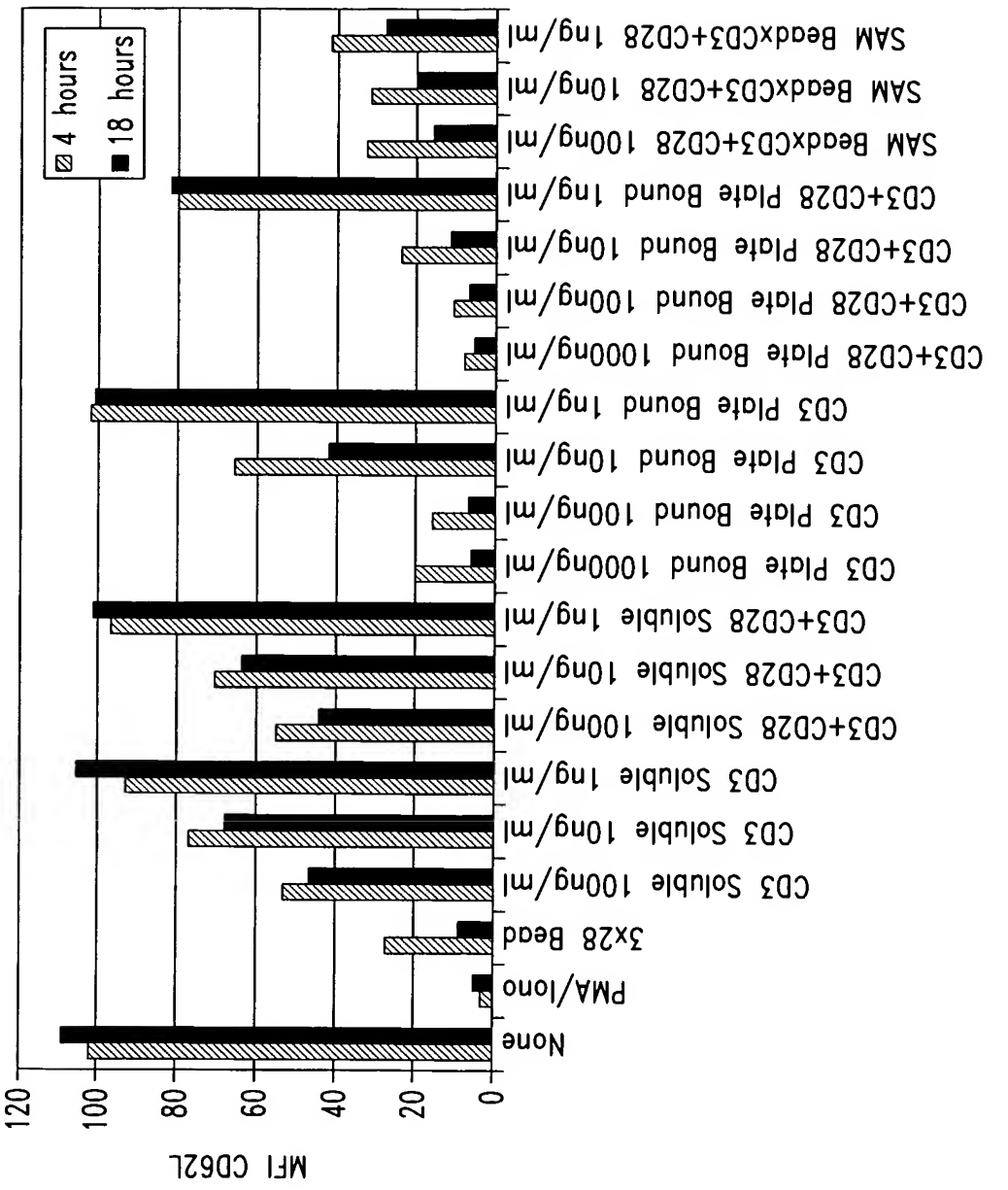

MFI CD62L
120
100
80
60
40
20
0
4 hours
18 hours
None
PMA/Iono
3x28 Bead
CD3 Soluble 100ng/ml
CD3 Soluble 10ng/ml
CD3 Soluble 1ng/ml
CD3+CD28 Soluble 100ng/ml
CD3+CD28 Soluble 10ng/ml
CD3+CD28 Soluble 1ng/ml
CD3 Plate Bound 1000ng/ml
CD3 Plate Bound 100ng/ml
CD3 Plate Bound 10ng/ml
CD3 Plate Bound 1ng/ml
CD3+CD28 Plate Bound 1000ng/ml
CD3+CD28 Plate Bound 100ng/ml
CD3+CD28 Plate Bound 10ng/ml
CD3+CD28 Plate Bound 1ng/ml
SAM BeadxCD3+CD28 100ng/ml
SAM BeadxCD3+CD28 10ng/ml
SAM BeadxCD3+CD28 1ng/ml

Fig. 26H

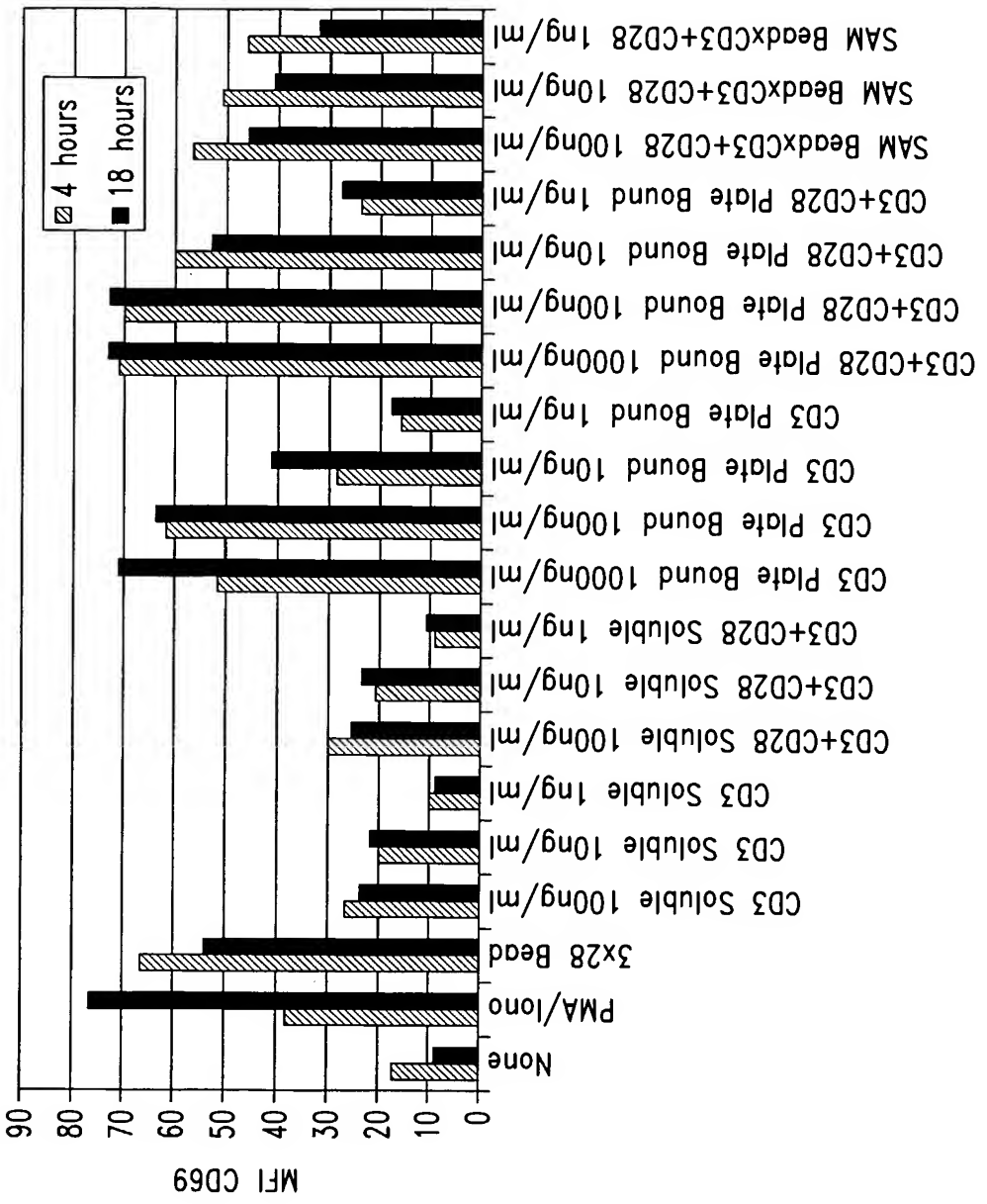

MFI CD69
0
10
20
30
40
50
60
70
80
90
4 hours
18 hours
None
PMA/Iono
3x28 Bead
CD3 Soluble 100ng/ml
CD3 Soluble 10ng/ml
CD3 Soluble 1ng/ml
CD3+CD28 Soluble 100ng/ml
CD3+CD28 Soluble 10ng/ml
CD3+CD28 Soluble 1ng/ml
CD3 Plate Bound 1000ng/ml
CD3 Plate Bound 100ng/ml
CD3 Plate Bound 10ng/ml
CD3 Plate Bound 1ng/ml
CD3+CD28 Plate Bound 1000ng/ml
CD3+CD28 Plate Bound 100ng/ml
CD3+CD28 Plate Bound 10ng/ml
CD3+CD28 Plate Bound 1ng/ml
SAM BeadxCD3+CD28 100ng/ml
SAM BeadxCD3+CD28 10ng/ml
SAM BeadxCD3+CD28 1ng/ml

Fig. 26I

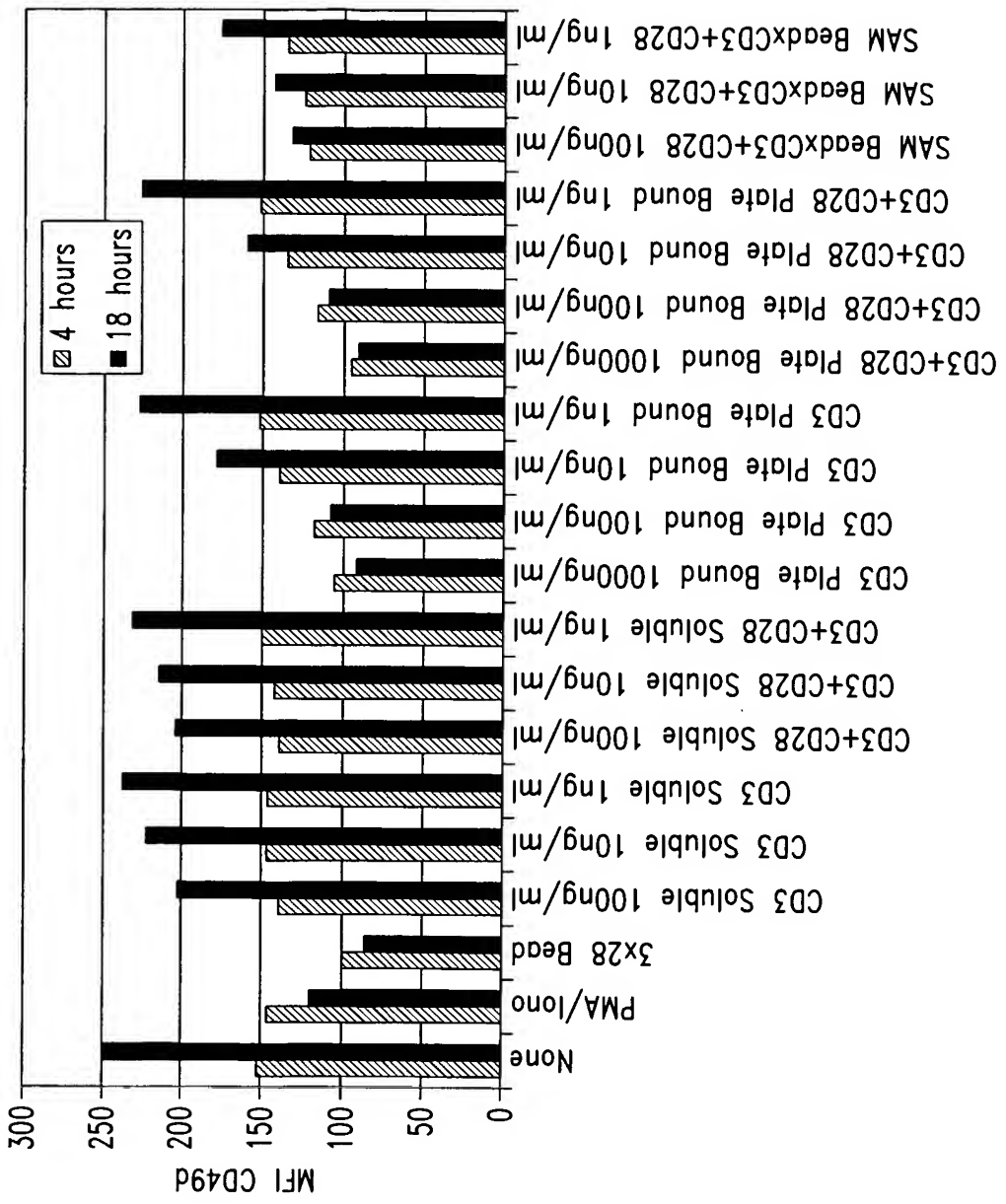

MFI CD49d
300
250
200
150
100
50
0
4 hours
18 hours
None
PMA/Iono
3x28 Bead
CD3 Soluble 100ng/ml
CD3 Soluble 10ng/ml
CD3 Soluble 1ng/ml
CD3+CD28 Soluble 100ng/ml
CD3+CD28 Soluble 10ng/ml
CD3+CD28 Soluble 1ng/ml
CD3 Plate Bound 1000ng/ml
CD3 Plate Bound 100ng/ml
CD3 Plate Bound 10ng/ml
CD3 Plate Bound 1ng/ml
CD3+CD28 Plate Bound 1000ng/ml
CD3+CD28 Plate Bound 100ng/ml
CD3+CD28 Plate Bound 10ng/ml
CD3+CD28 Plate Bound 1ng/ml
SAM BeadxCD3+CD28 100ng/ml
SAM BeadxCD3+CD28 10ng/ml
SAM BeadxCD3+CD28 1ng/ml

Fig. 26J

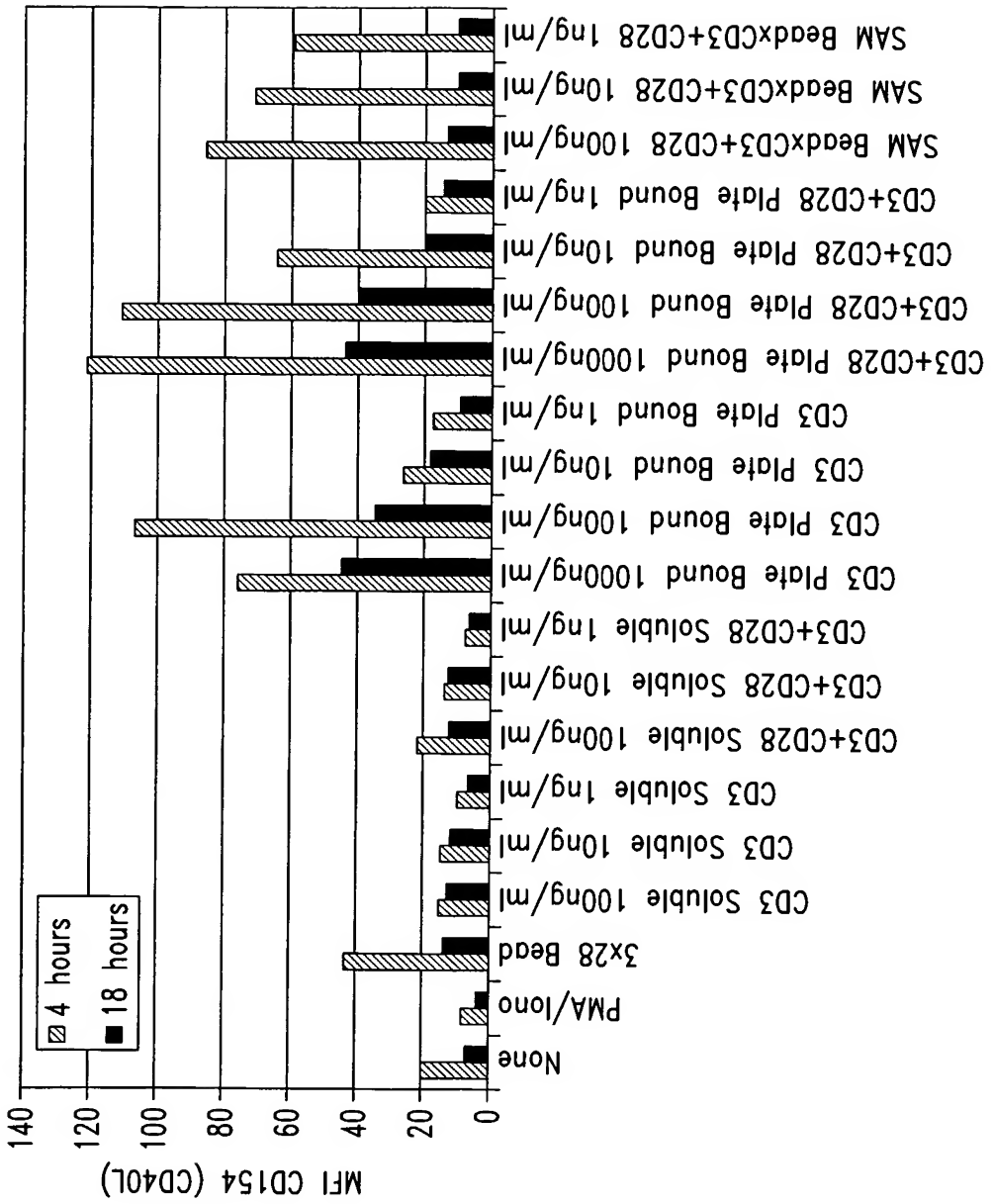

MFI CD154 (CD40L)
0
20
40
60
80
100
120
140
4 hours
18 hours
None
PMA/Iono
3x28 Bead
CD3 Soluble 100ng/ml
CD3 Soluble 10ng/ml
CD3 Soluble 1ng/ml
CD3+CD28 Soluble 100ng/ml
CD3+CD28 Soluble 10ng/ml
CD3+CD28 Soluble 1ng/ml
CD3 Plate Bound 1000ng/ml
CD3 Plate Bound 100ng/ml
CD3 Plate Bound 10ng/ml
CD3 Plate Bound 1ng/ml
CD3+CD28 Plate Bound 1000ng/ml
CD3+CD28 Plate Bound 100ng/ml
CD3+CD28 Plate Bound 10ng/ml
CD3+CD28 Plate Bound 1ng/ml
SAM BeadxCD3+CD28 100ng/ml
SAM BeadxCD3+CD28 10ng/ml
SAM BeadxCD3+CD28 1ng/ml

Fig. 26K

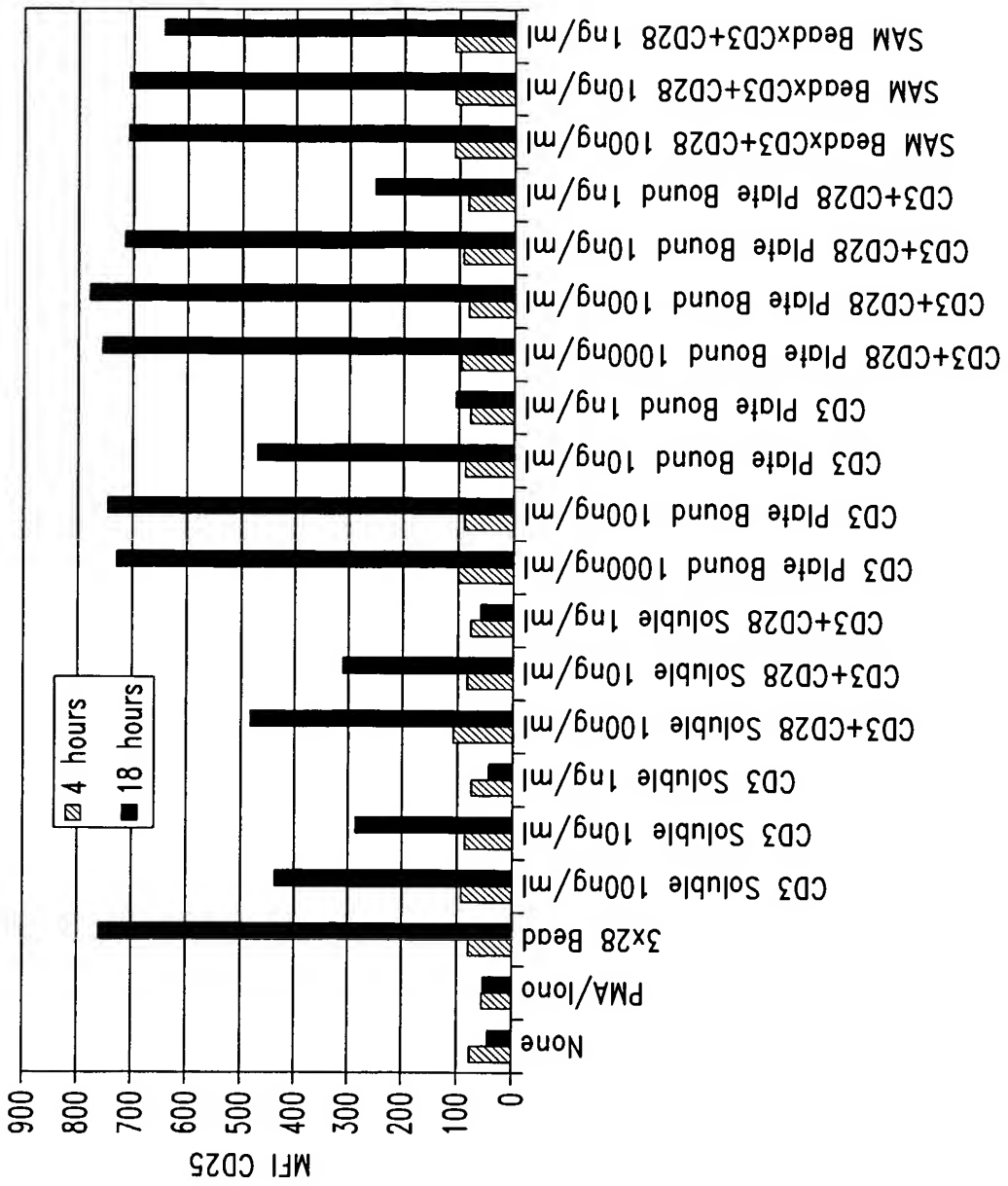

MFI CD25
0
100
200
300
400
500
600
700
800
900
4 hours
18 hours
None
PMA/Iono
3x28 Bead
CD3 Soluble 100ng/ml
CD3 Soluble 10ng/ml
CD3 Soluble 1ng/ml
CD3+CD28 Soluble 100ng/ml
CD3+CD28 Soluble 10ng/ml
CD3+CD28 Soluble 1ng/ml
CD3 Plate Bound 1000ng/ml
CD3 Plate Bound 100ng/ml
CD3 Plate Bound 10ng/ml
CD3 Plate Bound 1ng/ml
CD3+CD28 Plate Bound 1000ng/ml
CD3+CD28 Plate Bound 100ng/ml
CD3+CD28 Plate Bound 10ng/ml
CD3+CD28 Plate Bound 1ng/ml
SAM BeadxCD3+CD28 100ng/ml
SAM BeadxCD3+CD28 10ng/ml
SAM BeadxCD3+CD28 1ng/ml

Fig. 26L

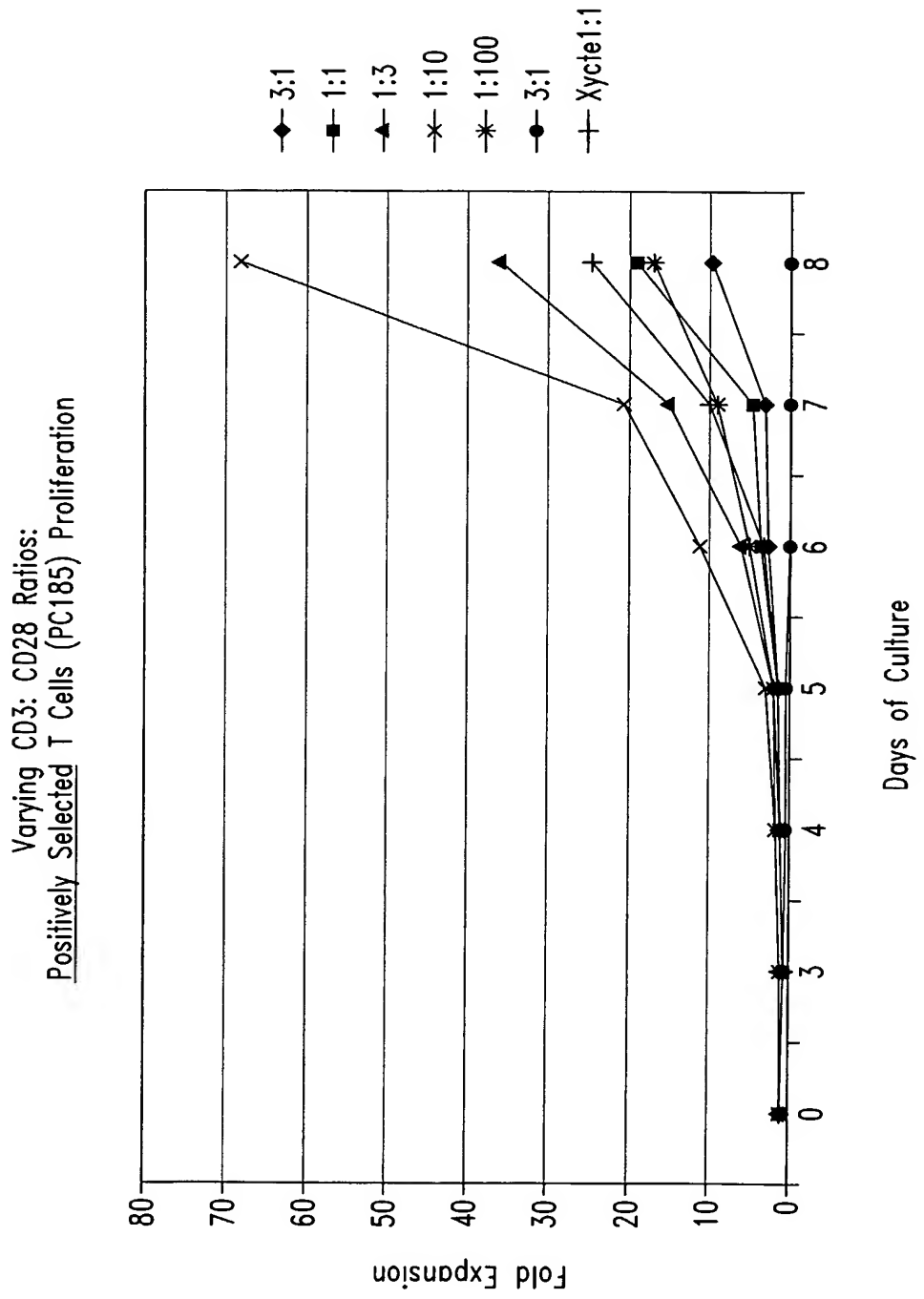

Varying CD3: CD28 Ratios:
Positively Selected T Cells (PC185) Proliferation
3:1
1:1
1:3
1:10
1:100
3:1
Xycte1:1
Fold Expansion
0
10
20
30
40
50
60
70
80
0
3
4
5
6
7
8
Days of Culture

Fig. 27

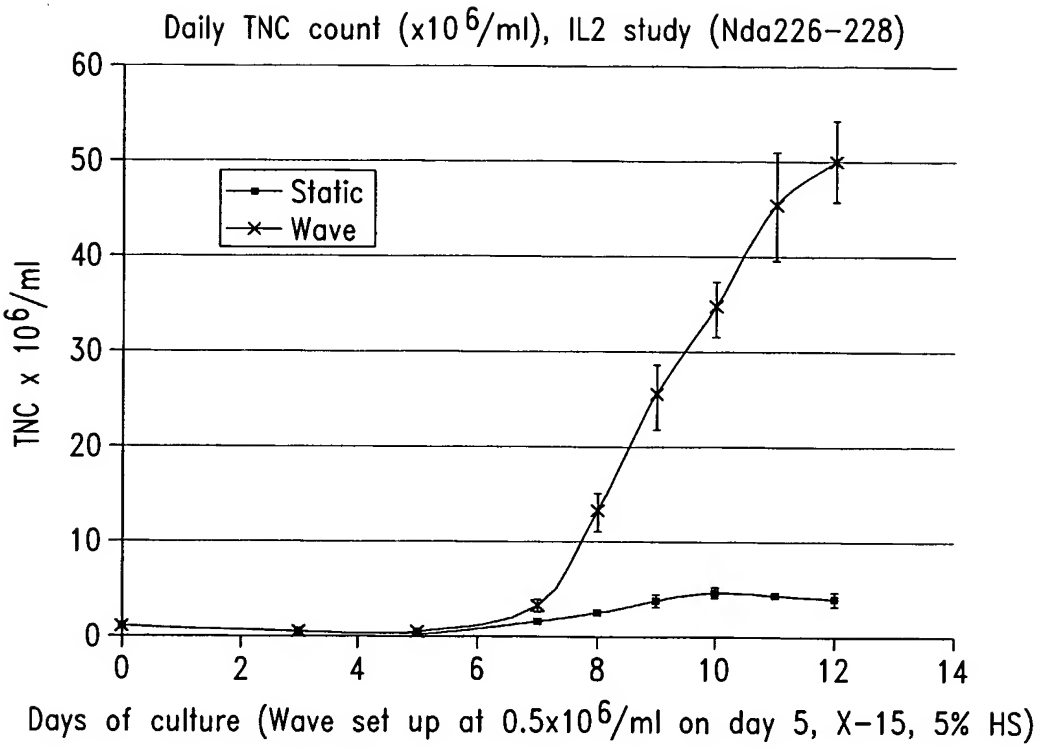

Daily TNC count (x10 6/ml), IL2 study (Nda226-228)
60
50
40
30
20
10
0
Static
Wave
TNC x 10 6/ml
0
2
4
6
8
10
12
14
Days of culture (Wave set up at 0.5x10 6/ml on day 5, X-15, 5% HS)

Fig. 28

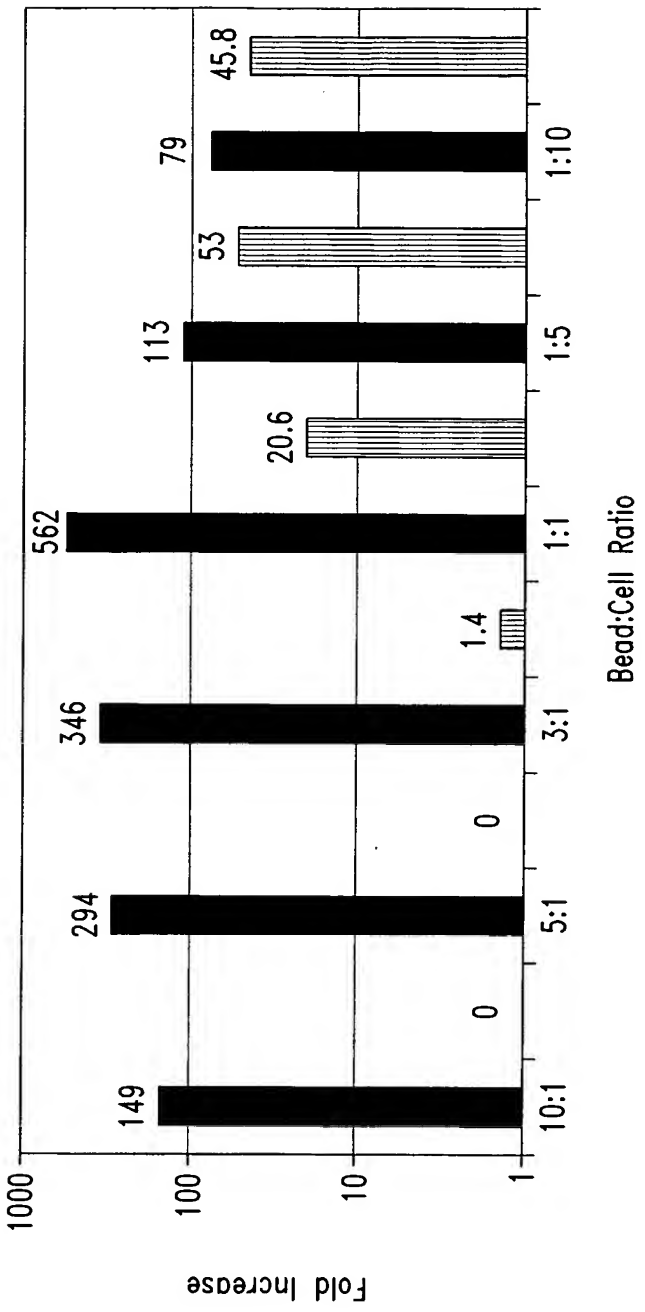

Fold Increase
1000
100
10
1
149
294
346
562
113
79
45.8
0
0
1.4
20.6
53
10:1
5:1
3:1
1:1
1:5
1:10
Bead:Cell Ratio

Fig. 29

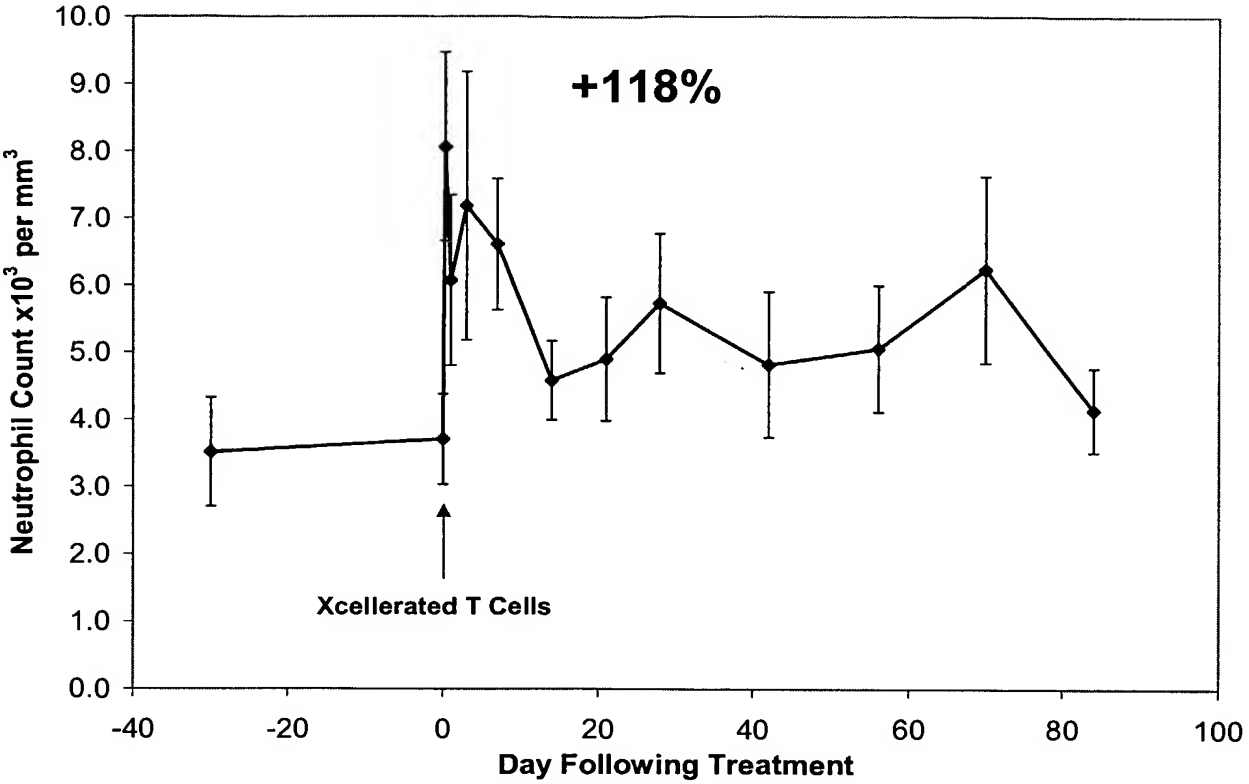


Fig. 30A

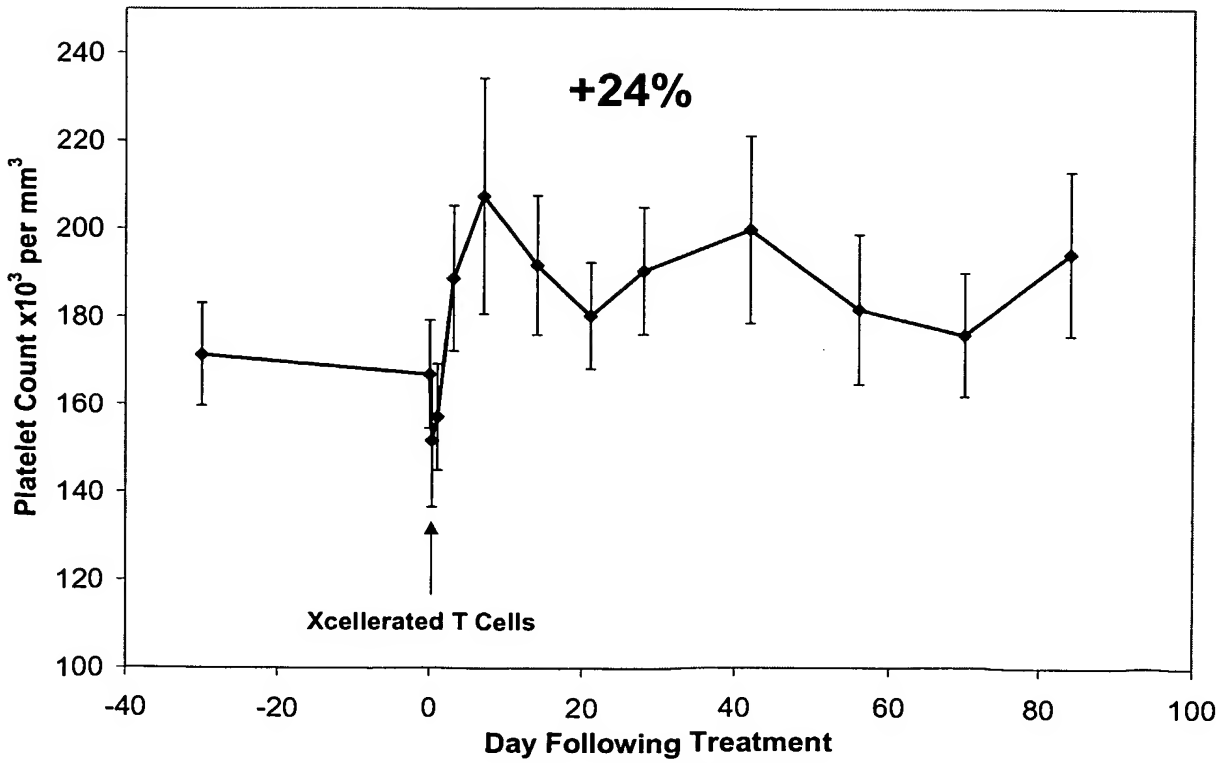


Fig. 30B

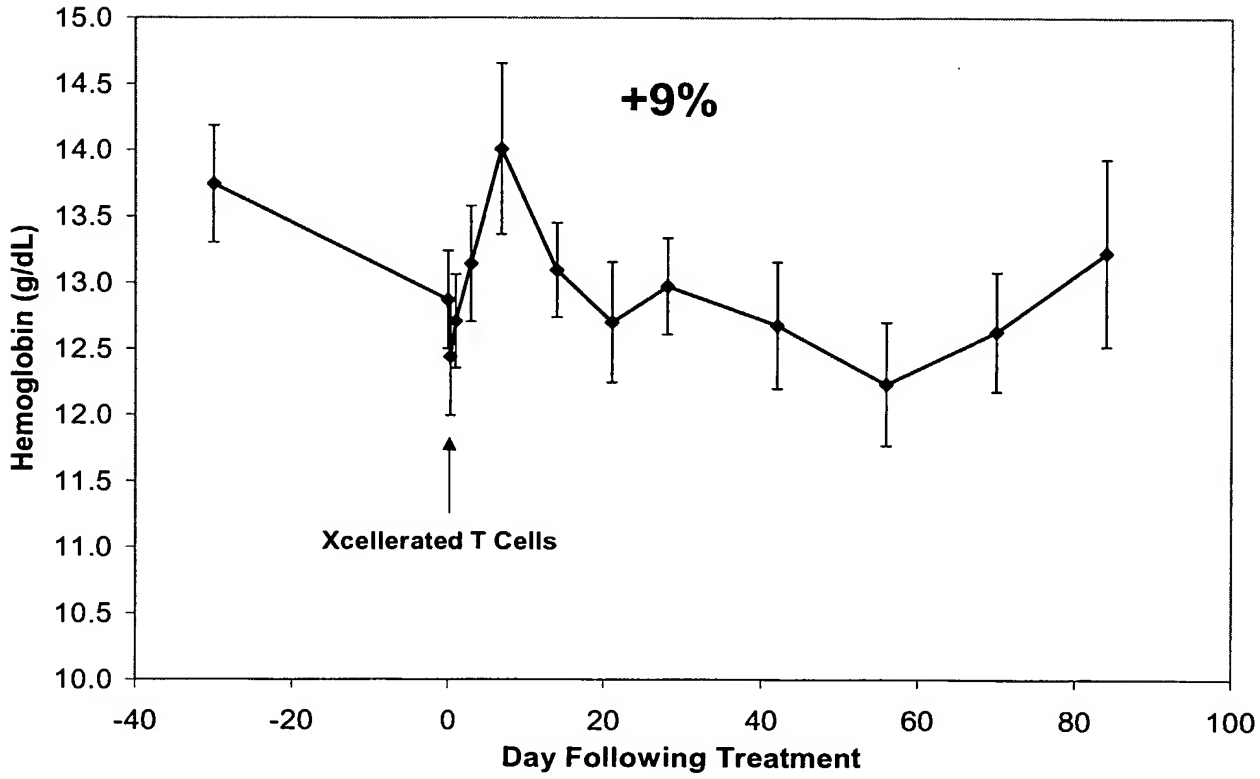

15.0
14.5
14.0
13.5
13.0
12.5
12.0
11.5
11.0
10.5
10.0
Hemoglobin (g/dL)
+9%
Xcellerated T Cells
-40
-20
0
20
40
60
80
100
Day Following Treatment

Fig. 30C

INTERNATIONAL SEARCH REPORT

Interna... ...pplication No
PCT/US2004/030895

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 A61K39/395 C12N5/06 A61P37/04

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 A61K C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, BIOSIS, WPI Data, PAJ

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	LAPORT GINNA G ET AL: "Adoptive transfer of costimulated T cells induces lymphocytosis in patients with relapsed/refractory non-Hodgkin lymphoma following CD34+-selected hematopoietic cell transplantation." BLOOD. 15 SEP 2003, vol. 102, no. 6, 15 September 2003 (2003-09-15), pages 2004-2013, XP002316311 ISSN: 0006-4971	1-8, 10-15
Y	the whole document	1-16

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
17 February 2005	07/03/2005

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31–70) 340–2040, Tx. 31 651 epo nl, Fax: (+31–70) 340–3016	Lechner, O

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Form PCT/ISA/210 (second sheet) (January 2004)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	THOMPSON JOHN A ET AL: "A phase I trial of CD3/CD28-activated T cells (Xcellerated T cells) and interleukin-2 in patients with metastatic renal cell carcinoma." CLINICAL CANCER RESEARCH : AN OFFICIAL JOURNAL OF THE AMERICAN ASSOCIATION FOR CANCER RESEARCH. 1 SEP 2003, vol. 9, no. 10 Pt 1, 1 September 2003 (2003-09-01), pages 3562-3570, XP002316312 ISSN: 1078-0432 abstract	1-16
Y	WO 01/62895 A (XCYTE THERAPIES, INC; BERENSON, RON; LAW, CHE; BONYHADI, MARK; SAUND,) 30 August 2001 (2001-08-30) abstract page 48, line 20 - page 49, line 8 page 50, paragraphs 2,3	1-16
A	LUM L G ET AL: "Immune modulation in cancer patients after adoptive transfer of anti-CD3/anti-CD28-costimulated T cells-phase I clinical trial." JOURNAL OF IMMUNOTHERAPY (HAGERSTOWN, MD. : 1997) 2001 SEP-OCT, vol. 24, no. 5, September 2001 (2001-09), pages 408-419, XP002957619 ISSN: 1524-9557 abstract	1-16
A	BRUNO ANTONIO ET AL: "Positive selection of CD34+ cells by immunoadsorption: factors affecting the final yield and hematopoietic recovery in patients with hematological malignancies and solid tumors." TRANSFUSION AND APHERESIS SCIENCE : OFFICIAL JOURNAL OF THE WORLD APHERESIS ASSOCIATION : OFFICIAL JOURNAL OF THE EUROPEAN SOCIETY FOR HAEMAPHERESIS. APR 2002, vol. 26, no. 2, April 2002 (2002-04), pages 103-110, XP001205215 ISSN: 1473-0502 abstract	11

-/--

4

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	FLENS MARCEL J ET AL: "Efficient expansion of tumor-infiltrating lymphocytes from solid tumors by stimulation with combined CD3 and CD28 monoclonal antibodies" CANCER IMMUNOLOGY IMMUNOTHERAPY, vol. 37, no. 5, 1993, pages 323-328, XP001204982 ISSN: 0340-7004 abstract	1-16
A	VENUPRASAD K ET AL: "Human neutrophil-expressed CD28 interacts with macrophage B7 to induce phosphatidylinositol 3-kinase-dependent IFN-gamma secretion and restriction of Leishmania growth." JOURNAL OF IMMUNOLOGY (BALTIMORE, MD. : 1950) 15 JUL 2002, vol. 169, no. 2, 15 July 2002 (2002-07-15), pages 920-928, XP002316458 ISSN: 0022-1767 abstract	11-16

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2004/030895

Box II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Although claims 1-16 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US2004/030895

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 0162895 A	30-08-2001	AU 4328801 A	03-09-2001
		BR 0108545 A	29-06-2004
		CA 2406864 A1	30-08-2001
		CN 1419597 T	21-05-2003
		EP 1257632 A1	20-11-2002
		JP 2004500095 T	08-01-2004
		WO 0162895 A2	30-08-2001
		US 2003124122 A1	03-07-2003
		US 2003119185 A1	26-06-2003
		US 2003235908 A1	25-12-2003
		US 2004241162 A1	02-12-2004
		US 2002058019 A1	16-05-2002
		US 2002119568 A1	29-08-2002
		ZA 200206666 A	20-02-2004
		ZA 200309093 A	30-06-2004